IDENTIFICATION AND CHARACTERIZATION OF THE TRANSPORTERS INVOLVED IN THE DISPOSITION OF PERFLUOROALKYL SUBSTANCES

By

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Wen Zhao

M.S., University of Huazhong university of Science and Technology, 2010

B.S., University of Huazhong university of Science and Technology, 2008

Submitted to the graduate degree program in Pharmacology and the Graduate Faculty of the University of Kansas Medical Center in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

Chair: Bruno Hagenbuch, Ph.D.

Udayan Apte, Ph.D.

Michele Pritchard, Ph.D.

Gregory Reed, Ph.D.

Gustavo Blanco, Ph.D.

Date Defended: September 19th, 2016

The dissertation committee for Wen Zhao certifies that this is the approved version of the following dissertation:

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Chair: Bruno Hagenbuch, Ph.D.

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ABSTRACT

Perfluoroalkyl substances (PFASs), including perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs), are persistent amphiphilic chemicals many of which are distributed ubiquitously in the environment and can be frequently detected in human serum. High doses of certain PFAS cause toxicities in animal models. Renal clearance and hepatic accumulation of PFASs can vary among different species, between genders in the same species and are also influenced by the carbon chain length of the individual PFCAs. In general, PFASs with shorter-chain length are eliminated more efficiently through the kidney whereas the longer-chain length PFASs tend to accumulate in liver. Although there were extensive studies published over the past decade regarding toxicities of PFASs in animal models and potential health risks in humans, the molecular mechanisms responsible for PFAS's disposition, such as the roles of specific transporters involved, have not been clearly addressed. Absorption, distribution and elimination of certain xenobiotics are largely influenced by transporters and recently published studies demonstrate that also PFCAs are substrates for several transporters. In order to further delineate the pharmacokinetic properties of the disposition of PFASs, the involvement of drug transporters expressed in liver, intestine and kidney was examined. In the first specific aim, I evaluated the hypothesis that drug transporters in the enterohepatic circulation contribute to the liver accumulation and long half-lives of long chain PFASs. To address this aim, uptake studies with perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonate (PFOS) using cells expressing liver

transporters NTCP or OATPs as well as intestinal transporters ASBT, OATPs or OSTα/β were performed. The results demonstrated that human and rat NTCP, human OATP1B1, OATP1B3, OATP2B1, OSTα/β and rat OATP1A1, OATP1B2, OATP2B1, OATP1A5 can transport all three PFSAs, whereas, human ASBT can only transport PFOS. In addition, inhibition studies with human MRP2, BCRP and BSEP containing Sf9 vesicles suggested that these efflux transporters might be involved in the canalicular secretion of PFHxS and/or PFOS in the liver. In the second specific aim, I evaluated the hypothesis that the differences of renal clearance of PFASs among different species and genders are due to the differences of specific transporters expressed in the kidney. To address this aim, rat renal transporters OAT1, OAT3 and OATP1A1 were examined for the transport of PFBS, PFHxS and PFOS. The results show that the three PFSAs are substrates of rat OAT1, OAT3 and OATP1A1. In summary, this dissertation reveals that 1) drug transporters expressed in the liver and the intestine and involved in the enterohepatic circulation of bile acids contribute to the long half-lives and the hepatic accumulation of PFHxS and PFOS in humans; 2) drug transporters in the liver, the intestine and the kidney contribute to species-, gender- and chain length-dependent elimination of PFASs.

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ABC	=	ATP-binding cassette
ANOVA	=	analysis of variance
ASBT	=	apical sodium bile acid transporter
BCRP	=	breast cancer resistance protein
BSEP	=	bile salt export pump
BSP	=	bromosulfophthalein
C10	=	perfluorodecanoate
C7	=	perfluoroheptanoate
C8	=	perfluorooctanoate
C9	=	perfluorononanoate
cAMP	=	cyclic adenosine monophosphate
CAR	=	constitutive androstane receptor
CCK8	=	cholecystokinin 8
CDCF	=	5(6)-carboxy-2’7’-dichiorfluorescein
cGMP	=	cyclic guanosine monophosphate
CHO	=	Chinese hamster ovary
CYP	=	cytochrome P-450
DHEAS	=	dehydroepiandrosterone sulfate
DMEM	=	Dulbecco’s modified eagle medium
DNA	=	deoxyribonucleic acid
FBS	=	fetal bovine serum
FDA	=	Food and Drug Administration

FXR	=	farnesoid X receptor
HEK293	=	human embryonic kidney cells clone 293
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF	=	hepatocyte nuclear factor
HPLC	=	high performance liquid chromatography
HSC	=	Human Subjects Committee
K_m	=	Michaelis constant
LXR	=	liver X receptor
MDCK	=	Madin-Darby canine kidney
MDR	=	multidrug resistance
mg	=	milligram
min	=	minute
mRNA	=	messenger ribonucleic acid
MRP	=	multidrug resistance-associated protein
MS	=	mass spectrometry
NSAID	=	nonsteroidal anti-inflammatory drug
NTCP	=	Na^+/taurocholate cotransporting polypeptide
OAT	=	organic anion transporter
OATP	=	organic anion transporting polypeptide
OCT	=	organic cation transporter
OCTN	=	organic zwitterion/cation transporter
OST	=	organic solute transporter
PBPK	=	physiologically based pharmacokinetic modeling

PCR	=	polymerase chain reaction
PFAS	=	perfluoroalkyl substances
PFBS	=	perfluorobutane sulfonate
PFCA	=	perfluoroalkyl carboxylates
PFDA	=	perfluorodecanoate
PFHpA	=	perfluoroheptanoate
PFHxA	=	perfluorohexanoate
PFHxS	=	perfluorohexane sulfonate
P-gp	=	P-glycoprotein
PFNA	=	perfluorononanoate
PFOA	=	perfluorooctanoate
PFOS	=	perfluorooctane sulfonate
PFSA	=	perfluoroalkyl sulfonates
pmol	=	picomole
PPAR	=	peroxisome proliferator-activated receptor
PXR	=	pregnane X receptor
SD	=	standard deviation
SHP	=	small heterodimer partner
SLC	=	solute carrier
SNP	=	single nucleotide polymorphism
SOAT	=	sodium-dependent organic anion transporter
V_{max}	=	maximum transport rate
WT	=	wild typ

CHAPTER 1

Introduction

I. Membrane transport proteins

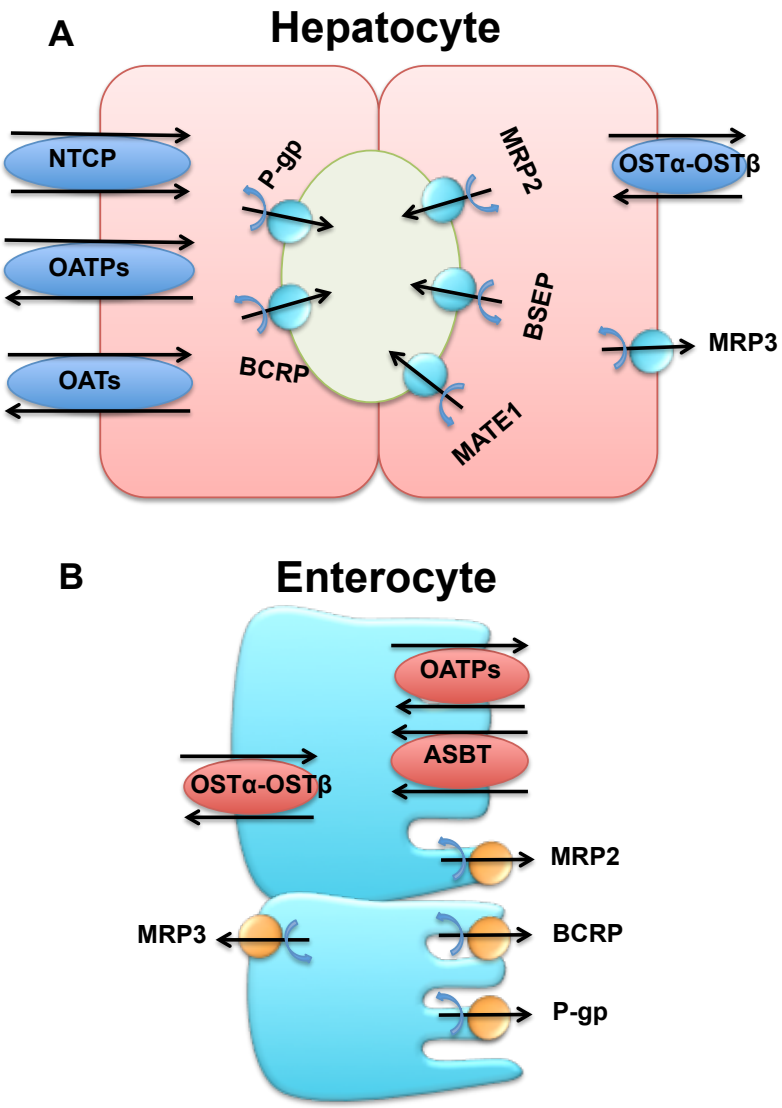
Cell membranes are lipid barriers surrounding each cell and very few molecules can cross the barrier without aid. Membrane transport proteins are proteins present in these cellular membranes that mediate the movement of ions, small molecules, or macromolecules across the membrane. Transporters can be divided, based on energy requirements, into passive or active. Passive transporters, or facilitated transporters, mediate transmembrane diffusion of solutes along their electrochemical gradient. While on the other hand, active transporters transport solutes against their gradient using energy either by using ATP directly or in the form of for example a sodium gradient. There are many types of membrane transporters, including ion channels, pores, ATPases and so on. The two big families of the ATP-binding cassette (ABC) transporters and the solute carriers (SLC) are of great importance in pharmacology (Giacomini et al., 2010).

Transporters play critical roles in the absorption, distribution and excretion of drugs and other xenobiotics. When it comes to the disposition of organic compounds, mainly the organic anion transporters (OATs) and organic cation transporters (OCTs) of the *SLC22* superfamily and the organic anion transporting polypeptides (OATPs) of the *SLCO* superfamily are involved. To a lesser extent,

members or the *SLC10* family can also mediate the influx of xenobiotics beyond their roles in bile acid transport. Considering the clinical importance of certain transporters with respect to the pharmacokinetics, safety and efficacy profiles of drugs, and the potential for drug-drug interactions, the FDA recommends or requires that transporters are being evaluated as a routine part of the drug development process (www.fda.gov/downloads/ drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf) (Giacomini et al., 2010).

Fig. 1-1 shows influx and efflux transporters expressed in hepatocytes and enterocytes that are important for the work presented in this dissertation. In the following, I will provide details regarding the *SLC10* and *SLCO* family focusing on their biological and toxicological roles. I will also briefly introduce OSTα-OSTβ, OATs and ABC transporters expressed in the liver (A) and intestine (B).

Figure 1-1: Selected SLC and ABC transporters expressed in hepatocytes and enterocytes



II. Introduction to SLC transporters

To date, 52 human SLC families with over 300 members have been identified. The HUGO Gene Nomenclature Committee (HGNC) website lists all of these genes (http://www.genenames.org/genefamilies/slc). At least a 20% amino acid identity is required for transporters to be assigned into the same family with a few exceptions. For instance, the *SLC51* family consists of *SLC51A* and *SLC51B*, which share no sequence identity but encode the two subunits for the same transporter, OSTα-OSTβ. The *SLC21* family is now named *SLCO*, made to accommodate a unique species-independent classification system (Hagenbuch and Stieger, 2013). In addition to humans, SLCs have been identified in many other species. Orthologues of human genes in other species usually use the same designation as the human gene (Hediger et al., 2013).

In this part of the introduction, I will focus on the biological, pharmacological and pharmaceutical perspectives of some members of the *SLC10*, *SLCO*, *SLC51* and *SLC22* families.

A. The *SLC10* family

1) Overview

The *SLC10* family was known as the "sodium bile salt cotransporting family" because two of its members are bile salt transporters. It is composed of seven members and three of them, including the Na^+/taurocholate cotransporting polypeptide (NTCP; *SLCO10A1*), the apical sodium-dependent bile salt

transporter (ASBT; *SLCO10A2*) and the sodium-dependent organic anion transporter (SOAT; *SLCO10A6*) have been functionally characterized (Claro da Silva et al., 2013). They utilize the out-to-in sodium gradient to mediate the co-transport of substrates into the cells. The major role of NTCP and ASBT is to facilitate the influx of bile acids in the liver and intestine to ensure the enterohepatic circulation of bile acids as digestive helpers and to ensure their uptake in other organs as signaling molecules. Besides bile acids, steroidal hormones and certain xenobiotics are also substrates of *SLC10* members. Instead of transporting classic bile acids, SOAT transports sulfated bile acids and steroid hormones (Claro da Silva et al., 2013).

2) NTCP

Rat and human NTCP were cloned in 1991 and 1994 (Hagenbuch et al., 1991; Hagenbuch and Meier, 1994), respectively, and have been extensively studied since then. NTCP orthologs in humans and rodents have 77% amino acid sequence identity. NTCP is expressed on the basolateral membrane of hepatocytes (Fig 1-1) to mediate the influx of mainly conjugated bile salts from the blood stream. Other substrates of NTCP include steroidal hormones such as estrone-3-sulfate and dehydroepiandrosterone sulfate (DHEAS), and a variety of xenobiotics such as bromosulfophthalein (BSP) and rosuvastatin (Stieger, 2011).

Expression of NTCP is regulated at both transcriptional and post-transcriptional levels. Due to the detergent properties of bile acids, their levels in the circulation and inside of cells are tightly regulated. In hepatocytes, high levels of bile acids

activate the nuclear receptor FXR, which down-regulates the expression of bile acid influx transporters (NTCP, ASBT and OATP1B1) via activation of SHP which leads to inhibition of HNF1α and the rate-limiting enzymes for bile acid synthesis (CYP7A1 and CYP27A1) (Eloranta and Kullak-Ublick, 2008). On the other hand, FXR activation up-regulates the expression of detoxifying enzymes and bile acid efflux transporters (Matsubara et al., 2013). OATP1B1 is a direct target of FXR, and FXR ligands increase OATP1B1 mRNA (Meyer Zu Schwabedissen et al., 2010). Besides FXR, other nuclear receptors, transcription factors as well as hormones also participate in the regulation of NTCP and ASBT. Furthermore, on the post-transcriptional level, mechanisms such as ubiquitination and protein kinase C-dependent phosphorylation are involved in NTCP and ASBT regulation (Claro da Silva et al., 2013). In addition to functioning as a transporter, NTCP is a receptor for hepatitis B and D virus, opening new opportunities for developing anti-HBV interventions (Yan et al., 2012; Yan et al., 2015).

3) ASBT

ASBT is localized at the apical membrane of the distal ileum (Fig 1-1), of cholangiocytes and of the renal proximal tubules (Dawson, 2011; Claro da Silva et al., 2013). The presence of ASBT in the distal ileum enables the circulation of bile acids between the liver and intestine, known as enterohepatic circulation. This enterohepatic circulation largely depends on the bile acid transporters NTCP the bile salt export pump (BSEP) and ASBT (Roberts et al., 2002). It relies on biliary secretion and intestinal reabsorption and is also observed for several solutes and certain drugs (Gao et al., 2014). As a consequence of the

enterohepatic circulation, the apparent half-life of a drug or other xenobiotics can be increased significantly. In the kidney, ASBT serves as a salvage transporter to prevent the loss of bile acids from the glomerular filtration. Due to the efficient recycling from small intestine and kidney, less than 10% of bile acids are excreted from the body every day. The expression of ASBT in the biliary tract together with bile acid transporters in hepatocytes is the foundation for another circulation within the liver named cholehepatic shunt (Benedetti et al., 1997; Meier and Stieger, 2002).

In contrast to NTCP, the spectrum of ASBT substrates is considered narrow. Initially, it was thought that ASBT can only transport taurine- or glycine-conjugated and unconjugated bile acids (Dawson, 2011). However, recent evidence shows that it is capable of transporting bile acid analogs (Kolhatkar and Polli, 2012). Conjugates at C-3 or C-7 were synthesized and tested for uptake studies. It turned out that a C-3 but not a C-7 modification of bile acids still afforded translocation by NTCP and ASBT. These results suggest that it should be possible to target ASBT and NTCP by conjugating drugs to the C-3 hydroxyl group of bile acids for therapeutic purposes.

Since ASBT is critical for maintaining bile acid homeostasis, it is important to know if a drug could influence its function or expression. Several studies have shown that the expression level of ASBT mRNA increased in the kidney and ileum with vitamin D treatment in response to the activation of the vitamin D

receptor (Chow et al., 2010; Chow et al., 2011). Another study showed that mice treated with ampicillin exhibited elevated ASBT levels due to the disturbance of the intestinal flora which produce secondary bile acids (Miyata et al., 2011). Furthermore, Zheng et al. demonstrated, using MDCK cells stably expressing ASBT, that statins and dihydropyridine calcium channel blockers are potent inhibitors of ASBT (Zheng et al., 2010). These results suggest that there indeed is a potential for drug-drug interaction at ASBT *in vivo*.

ASBT is an emerging therapeutic target for dyslipidemia, cholestasis, diabetes and chronic constipation. When ASBT is inhibited, bile acid reabsorption from the ileum is impeded and therefore the liver uses more cholesterol to make bile acids. Several ASBT inhibitors have been tested for their lipid lowering effects. For instance, a newly synthesized ASBT inhibitor, Polyacrylic acid-tetraDOCA conjugate, prevents the increase of cholesterol in the blood as well as triglyceride in the liver in mice fed a high fat diet (Park et al., 2015). A selective inhibitor of ASBT, A4250, attenuates cholestatic liver and bile duct injury in mice by reducing biliary bile acid concentrations (Baghdasaryan et al., 2016). In addition, 264W94, a first generation ASBT inhibitor, is a possible new treatment for type 2 diabetes (Chen et al., 2012). More recently, GSK2330672 was discovered as a highly potent, non-absorbable ASBT inhibitor, which lowers glucose in diabetic animals. It shows excellent develop-ability properties for the treatment of patients with type 2 diabetes (Wu et al., 2013). Last but not the least, A3309, a minimally absorbed ASBT inhibitor, increases stool frequency and improves constipation-

related symptoms in patients; these effects were maintained over 8 weeks of treatment. (Chey et al., 2011).

B. The *SLCO* superfamily

1) Overview

Besides sodium-dependent transporters, sodium-independent transporters, such as OATPs, also mediate the influx of bile acids into cells. OATPs are encoded by *SLCO* genes.

Rat OATP1A1 was the first OATP that was cloned (Jacquemin et al., 1994) and the first human OATP, OATP1A2 was cloned a year later (Kullak-Ublick et al., 1995). Until now, more than 300 members of the OATP superfamily have been identified and/or predicted from over 40 species, such as human, monkey, dog, pig, rat, mouse and fish. Among all of them, human and rodent OATPs are the most extensively studied and characterized. Based on their amino acid similarities, human and rodent OATPs are classified into 6 families, namely OATP1, OATP2, OATP3, OATP4, OATP5 and OATP6. Within each family, there can be subfamilies. For example, the OATP1 family contains the subfamilies OATP1A, OATP1B and OATP1C (Hagenbuch and Stieger, 2013).

2) Distribution and substrate specificity

Among all the human and rat SLCO superfamily members, the OATP1 and OATP2 family members are the most well-characterized. Table 1-1 lists the

localization and representative substrates of human and rat OATP1 and OATP2 family members. OATP1A2 is the only human OATP1A subfamily member while in rats 5 members have been identified. In contrast to the OATP1A subfamily, the OATP1B subfamily contains only one rodent (OATP1B2) but two human members, OATP1B1 and OATP1B3 (Hagenbuch and Stieger, 2013).

Unlike NTCP and ASBT, OATPs mediate the transport of a broad spectrum of structurally diverse compounds with overlapping substrate specificities as indicated in Table 1-1 (Roth et al., 2012; Hagenbuch and Stieger, 2013). Bile salts, steroid and thyroid hormones are common endogenous substrates of OATPs with the exception that OATP2A1 mainly transports prostaglandins. Additionally, many xenobiotics, including numerous drugs, are substrates of OATPs. This can lead to drug-drug and drug-food interactions, like the interaction between statins and grapefruit juice (Lilja et al., 2004; Kalliokoski and Niemi, 2009).

Table 1-1: Brief summary of the OATP1 and OATP2 subfamily members

Subfamily	Species	Transporter	Distribution	Selected substrates
OATP1A	Human	OATP1A2	Brain, liver, lung, testis	Bile salts Steroid hormones Thyroid hormones Prostaglandin E2 Bilirubin Epigallocatechin gallate Statins
	Rat	OATP1A1	Liver, kidney, brain	
		OATP1A3	Kidney	
		OATP1A4	Liver, brain, eye	
		OATP1A5	Intestine, brain	
		OATP1A6	Kidney	
OATP1B	Human	OATP1B1	Liver	Bile salts Bilirubin Steroid hormones Thyroid hormones Prostaglandine E_2 Bromosulphophthalein (BSP) Statins Rifampicin Valsartan
		OATP1B3	Liver	Bile salts Bilirubin Steroid hormones Thyroid hormones CCK8 Digoxin Epigallocatechin gallate Rifampicin Statins Paclitaxel
	Rat	OATP1B2	Liver	Bile salts Steroid hormones CCK8 Prostaglandine E_2 Thyroids
OATP1C	Human	OATP1C1	Brain, testis	BSP Steroid hormones Thyroid hormones Taurocholate
	Rat	OATP1C1	Brain, eye	CCK8 Steroid hormones Thyroid hormones
OATP2A	Human	OATP2A1	Ubiquitous	Prostaglandins
	Rat	OATP2A1	Ubiquitous	Prostaglandins
OATP2B	Human	OATP2B1	Liver, placenta, intestine, eye	Bile salts Steroid hormones Thyroid hormones BSP Statins
	Rat	OATP2B1	Liver, lung, intestine	Taurocholate Prstaglandins Thromboxane B_2

3) Regulation of expression

Transcriptional regulation is largely involved in the control of OATP expression. Expression of OATP1B1 and OATP1B3 in hepatocytes is controlled by hepatocyte nuclear factor 1α (HNF1α) (Jung et al., 2001) and HNF3β (Vavricka et al., 2004), respectively. In addition, other transcription factors like PXR, FXR and LXR play roles in the regulation of OATP1B1 expression (Meyer Zu Schwabedissen et al., 2010; Suzuki et al., 2011). Furthermore, PXR and CAR regulate the expression of OATP1A2 (Meyer zu Schwabedissen et al., 2008).

Post-translational regulation also occurs for OATPs. The C-terminal end of OATP1A2 can interact with PDZ proteins, potentially leading to its membrane localization (Kato et al., 2004). Phosphorylation affects membrane expression of rat OATP1A1 (Choi et al., 2011). In addition, activation of protein kinase C regulates the function of OATP1B1, OATP1B3 and OATP2B1 via triggering the rapid internalization and recycling of these transporter proteins. (Kock et al., 2010; Powell et al., 2014; Hong et al., 2015).

4) Clinical significance

So far, only a few diseases have been linked to OATP function. One example is Rotor syndrome, a rare, benign syndrome presenting with unconjugated and conjugated hyperbilirubinemia. It results from disruption of hepatic uptake of bilirubin glucuronide due to complete functional OATP1B1 and OATP1B3 deficiency (van de Steeg et al., 2012). However, many studies have shown that

altered OATP expression levels resulting from single-nucleotide polymorphisms (SNPs) and/or allele deletion can disturb normal pharmacokinetics. Various polymorphisms of OATP1B1 have been identified and characterized. Two common SNPs are the *SLCO1B1* 388A>G and 521T>C alleles, which can form four distinct haplotypes, including **1A* (c.388A-c.521T), **1B* (c.388G-c.521T), **5* (c.388A-c.521C), and **15* (c.388G-c.521C) (Niemi et al., 2011). Presence of *SLCO1B1*15* can lead to a higher risk for developing severe hyperbilirubinemia in neonates (Buyukkale et al., 2011) and to higher serum bilirubin levels in adults (Ieiri et al., 2004). Carriers of *SLCO1B1*5* and **15* haplotypes, individuals with these haplotypes have increased plasma levels of drugs such as pravastatin (Nishizato et al., 2003), pitavastatin (Chung et al., 2005), and atrasentan (Katz et al., 2006) due to reduced transport function of these mutant OATP1B1 proteins. Given the importance of OATPs expressed in hepatocytes for drug elimination, the FDA requires OATP-inhibition studies for all drugs, and, for certain drugs, even uptake studies, following the recommendations of The International Transporter Consortium (Giacomini et al., 2010).

C. Brief introduction to OSTα-OSTβ (*SLC51A* family)

The organic solute transporter alpha-beta (OSTα-OSTβ) is one of the most unique members in the SLC family because the functional transporter consists of two different proteins. It was cloned from the liver of the little skate (*Leuroraja erinacea*) by Wang et al in 2001 (Wang et al., 2001). The mouse and human orthologs were subsequently cloned and characterized by Seward et al. in 2003

(Seward et al., 2003). OSTα is encoded by the *SLC51A* gene, has 340-amino acids and is predicted to have a 7- transmembrane domain structure. OSTβ is a 128-amino acid single-transmembrane domain polypeptide and encoded by *SLC51B*. In contrast to other organic anion transporters, heterodimerization of OSTα and OSTβ is required for transport activity.

OSTα and OSTβ are ubiquitously expressed throughout the body but highest expression levels are found in tissues involved in bile acid and steroid homeostasis, such as intestine, liver (Fig 1-1) and kidney. Depending on the concentration gradient, OSTα-OSTβ is capable of mediating influx as well as efflux of substrates along their gradient. OSTα-OSTβ is a key player in the enterohepatic circulation. At the basolateral membrane of enterocytes, OSTα-OSTβ is responsible for the excretion of bile acids reabsorbed by ASBT from the ileum into the portal circulation. OSTα-OSTβ is a multispecific transporter and substrates besides bile acids include estrone-3-sulfate, digoxin, prostaglandin E2 and DHEAS. (Ballatori et al., 2009; Ballatori et al., 2013)

D. Brief introduction to OATs (*SLC22A* family)

The SLC22A family contains transporters for organic anions (OATs), organic cations (OCTs) and zwitterions (organic zwitterions/cation transporters (OCTNs)) (Koepsell, 2013). OATs are multispecific transporters mainly localized in the kidney where they mediate the excretion with the exception of OAT4, which mediates the reabsorption of organic anions, including endogenous solutes and

numerous xenobiotics including drugs. Compared to OATPs, which mainly transport large hydrophobic organic anions, substrates of OATs are smaller and more hydrophilic organic anions (Roth et al., 2012).

At present, no crystal structure exists for any of the OATs. However, based on amino acid sequence analysis and homology modeling, OATs have a predicted structure consisting of 12 α-helical transmembrane domains, a large extracellular loop between transmembrane domain 1 and 2 as well as a large intracellular loop between transmembrane domain 6 and 7. Among the different OATs, OAT1, OAT2, OAT3 and OAT4 are well characterized (Koepsell, 2013).

The first mammalian OAT, rat OAT1 was cloned in 1997 (Sweet et al., 1997), the first human OAT1 was subsequently identified in the following year (Reid et al., 1998). The main expression site of OAT1 is at the basolateral membrane of proximal tubules in the kidney, mediating the secretion of organic anions. To a lesser extent, OAT1 is also expressed in the skeletal muscle and choroid plexus. Endogenous substrates of OAT1 include medium chain fatty acids, α-ketoglutarate, cAMP and cGMP, prostaglandins, and urate. In addition, OAT1 can transport various drugs like the antibiotic tetracycline and antiviral drugs such as acyclovir and adefovir (Rizwan and Burckhardt, 2007; Burckhardt, 2012).

OAT2 is mainly found at the basolateral membrane of the liver. In the kidney, human OAT2 is also expressed at the basolateral membrane of proximal tubules

while rodent OAT2 is localized at the apical membrane. OAT2 transports endogenous compounds such as glutamate, urate, purine and pyrimidine nucleobases, nucleosides and nucleotides. Many drugs including salicylate, bumetanide and tetracycline are also substrates of OAT2 (Rizwan and Burckhardt, 2007; Burckhardt, 2012).

Along with OAT1, OAT3 is highly expressed at the basolateral membrane of proximal tubule cells. OAT3 mRNA is also detected in the spinal cord, choroid plexus, eye and adrenal gland. Among all the OATs expressed in human kidney (OAT1-4), OAT3 is the most abundantly expressed (Motohashi et al., 2002). OAT3 transport is coupled to the efflux of α-ketoglutarate. Endogenous substrates of OAT3 include cAMP, cortisol, prostaglandins, DHEAS, estrone-3-sulfate, taurocholate and urate. Drugs that can be transported by OAT3 include β-Lactam-antibiotics, H2 antagonists, antiepileptic, cytostatics, diuretics, NSAIDs, statins and uricosurics (Rizwan and Burckhardt, 2007; Burckhardt, 2012).

OAT4 is only expressed in humans. It is located at the apical membrane of proximal tubule epithelial cells and at the placental epithelial basolateral membrane. Human OAT4 transports conjugated hormones such as estrone-3-sulfate, DHEAS, and prostaglandins. Drugs that interact with OAT4 are very similar to the drugs that interact with OAT1-3, including antibiotics, diuretics, statins and antivirals (Rizwan and Burckhardt, 2007).

E. Brief introduction to ABC transporters in the liver and intestine

ABC transporters use energy from ATP hydrolysis to move a wide range of molecules out of mammalian cell membranes as well as intracellular membranes against their electrochemical gradient. These proteins are encoded by ABC gene. Fourty-nine ABC genes exist in humans, classified into seven subfamilies, from ABCA to ABCG. Substrates of ABC transporters include metal ions, peptides, amino acids, sugars and a large number of hydrophobic compounds and metabolites (Vasiliou et al., 2009).

Together with influx transporters, ABC transporters facilitate the movement of solutes across the enterocytes and hepatocytes by mediating their efflux. As shown in Fig 1-1, at the canalicular membrane of hepatocytes, ABC pumps including P-glycoprotein (P-gp), BSEP, multidrug resistance associated protein 2 (MRP2), and breast cancer resistance protein (BCRP) are expressed, while MRP3 is located at the sinusoidal membrane. In enterocytes, P-gp, MRP2 and BCRP are found at the apical membrane and MRP3 is expressed at the basolateral membrane. Table 1-2 summarizes the encoding genes, tissue distribution and substrate specificities of these transporters.

Table 1-2: Brief summary of ABC transporters in hepatocytes and enterocytes (Dean et al., 2001)

Transporter	Gene	Tissue	Substrates
P-gp/MDR1	*ABCB1*	Intestine Liver Kidney Placenta BBB	Neutral and cationic organic compounds, drugs (e.g. vinblastine, doxorubicin, paclitaxel, methotrexate, losartan)
BSEP	*ABCB11*	Liver	Bile salts
MRP2	*ABCC2*	Intestine Liver Kidney	Non-bile salt organic anions, drugs (e.g. vinblastine, doxorubicin, methotrexate, glutathione conjugates)
MRP3	*ABCC3*	Intestine Liver Kidney Pancreas Adrenal glands	Glucuronide conjugates, bile salts, Methotrexate, doxorubicin
BCRP	*ABGG2*	Liver Placenta Breast	Organic anions, chemotherapeutics

P-gp was first demonstrated to confer resistance to colchicine in CHO cells (Juliano and Ling, 1976) and then was shown to confer resistance to a wide range of amphiphilic drugs. Substrates of P-gp are usually bulky, hydrophobic organic cations. Expressed at the apical membranes of epithelial cells, it has great influence on drug disposition by limiting absorption in the intestine and enhancing urinary and biliary excretion in the kidney and liver, respectively. The drug resistance role of P-gp expressed in the placenta and at the blood-brain-barrier largely contributes to the protection mechanism of these important compartments (Honjo et al., 2002; Kock and Brouwer, 2012).

In contrast to P-gp, BSEP has a narrow substrate spectrum and mainly transports bile salts. BSEP is exclusively expressed at hepatocyte canalicular membrane, where it is responsible for the excretion of bile acids into the bile ducts and therefore constitutes the major driving force for the generation of bile salt-dependent bile flow (Stieger, 2011).

Two ABCC subfamily members, MRP2 at the apical membrane and MRP3 at the basolateral membrane, are usually found in epithelial cells, especially in the intestine, liver and kidney. Organic anions are common substrates of MRPs while they can also mediate the efflux of certain uncharged amphipathic, or even cationic compounds (Slot et al., 2011).

ABCG genes encode half-transporters that must form homo- or heterodimers for function (Vasiliou et al., 2009). BCRP is the only member of this subfamily that is

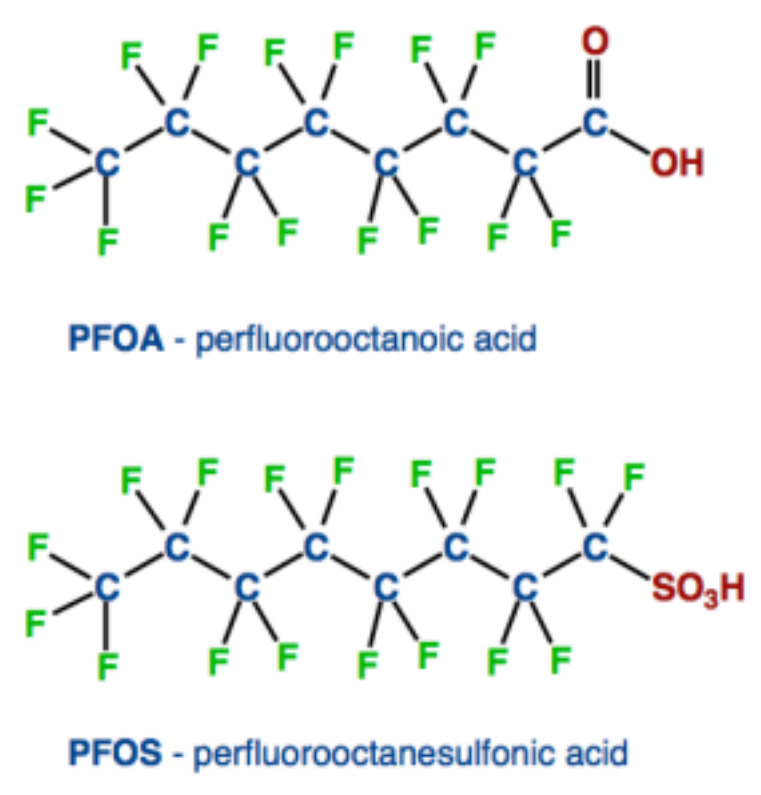
involved in drug transport. Similar to P-gp, BCRP was initially identified in drug-resistant cancer cell lines and then was found to locate to the apical membrane of epithelial cells in many organs protecting them from toxicants. Numerous chemotherapeutics and hydrophobic organic anions are substrates of BCRP.

III. Introduction to perfluoroalkyl substances (PFASs)

A. Terminology and classification

Perfluoroalkyl substances (PFASs) are defined as aliphatic substances for which all of the H atoms on the carbon backbone are replaced by F atoms, except those H atoms whose substitution would modify the nature of any functional groups present (Buck et al., 2011). Depending on the functional group, PFASs are divided into the two families of the perfluoroalkyl carboxylates (PFCAs) and the perfluoroalky sulfonates (PFSAs). Within each family, PFASs are frequently referred to as “long-chain” or “short-chain”. Defined by the Organization for Economic Co-operation and Development, “long-chain” refers to PFCAs with 8 or more carbons, and to PFSAs with 6 or more carbons (Buck et al., 2011). Fig. 1-2 shows two examples of long-chain PFASs, perfluorooctanoic acid (PFOA) with 7 fluorinated carbons and one additional carbon in the functional group, and perfluorooctanesulfonic acid (PFOS) with 8 fluorinated carbons.

Figure 1-2: Examples of long-chain PFCAs and PFSAs



B. Environmental contamination by PFASs.

Since the 1940s, precursors of PFASs or PFASs themselves have been widely used in the industry as surfactants due to their extremely stable Carbon-Fluorine bonds. PFOS and PFOA are ubiquitously distributed in the environment. Once released into the environment, some higher molecular weight PFASs degrade to PFCAs and PFSAs, primarily to PFOA and PFOS (Buck et al., 2011). On the other hand, the strong Carbon-Fluorine bond also makes PFCAs and PFASs highly resistant to degradation and therefore persistent in the environment. Since the first reported global distribution of PFOS in wildlife liver and blood (Giesy and Kannan, 2001), increasing amounts of samples collected from biota and humans have been analyzed. To date, data are available for the distribution of certain members of the PFASs, mainly for PFOS, PFOA, PFHxS and some others, in surface water, sediments, wildlife as well as in humans from many countries (Higgins et al., 2005; Houde et al., 2011). Since these compounds can migrate via air and water, PFOA and PFOS are even detectable in the most remote oceanic areas and are even found in polar bears (Giesy and Kannan, 2001; Smithwick et al., 2005). Production of PFOS and PFOA was phased-out in the USA between 2000 and 2002 and short-chain PFASs were developed to replace the phased-out compounds.

C. Concentrations and half-lives of PFASs in humans

PFOS, PFHxS, perfluorononanoic acid (PFNA) and PFOA are found in nearly all people tested. According to the National Health and Nutrition Examination

Survey (NHANES) 2003-2004, the range of serum PFOS, PFOA, PFHxS and PFNA was 0.4 µg/L to 435 µg/L, 0.1 µg/L to 77.2 µg/L, 0.3 µg/L to 82.0 µg/L and 0.1 µg/L to 11.5 µg/L, respectively, in the general U.S. population (Calafat et al., 2007). Results of the survey also indicated that concentrations of PFOS, PFOA and PFHxS are significantly higher in males than in females. In comparison with the general population, occupational workers have much higher serum concentrations of PFOS (145-3490 ng/ml), PFHxS (16-1295 ng/ml) and PFOA (72-5100 ng/ml) (Olsen et al., 2007). Furthermore, a comparison of serum concentrations of PFASs in children demonstrated that, in general, children have higher serum concentrations than adolescents and adults (Olsen et al., 2004; Kato et al., 2009b). It was suggested that this might be due to a unique exposure pattern for children, such as e.g. playing on carpets treated with PFASs. The main routes of human exposure include oral, through dietary intake and drinking water, and by inhalation from consumer products, indoor air and/or dust (Begley et al., 2005; Emmett et al., 2006; Kato et al., 2009a; Noorlander et al., 2011; Domingo et al., 2012). Since 1999-2000, serum levels of PFOS and PFOA have decreased gradually in U.S. residents (Centers for Disease Control and Prevention, 2015). Serum elimination half-lives of PFASs in humans are very long. Olsen et al. estimated from retired fluorochemical workers that the mean serum elimination half-lives for PFOS, PFHxS and PFOA are 4.6, 7.1 and 3.4 years, respectively (Olsen et al., 2007). The short-chain PFBS is estimated to have a half-life of 26 days from 6 human subjects (Olsen et al., 2009). Further analysis is needed for a more accurate evaluation of the half-lives of PFASs in

humans and for comparison between different populations, such as between males and female as well as among children, adults and elderly.

D. Toxicities of PFASs

Exposure to high doses of PFOA increased the incidence of hepatocellular adenomas, thyroid follicular cell adenomas, Leydig cell tumors, and pancreatic acinar cell tumors in rats (Lau et al., 2007; Andersen et al., 2008). In non-human primates, repeated high doses of PFOS treatment showed reduced body weight, increased liver weight and reduced cholesterol levels while no significant adverse effects was observed in monkeys treated with lower doses (Seacat et al., 2002). Other toxic effects, such as developmental toxicity (Lau et al., 2004; Lau et al., 2007), immune depression (Corsini et al., 2014), altered weight gain after in utero exposure (Hines et al., 2009) and thyroid hormone disruption (Lau et al., 2007), were also observed with high dose of PFOS. Epidemiological studies found a direct association between PFOS and PFOA levels and serum cholesterol levels in humans (Frisbee et al., 2010; Nelson et al., 2010). An association between PFOA levels and kidney and testicular cancer among adults who lived in the Mid-Ohio Valley and were exposed to PFOA in the drinking water due to chemical plant emissions was also indicated (Barry et al., 2013).

No genotoxic effect has been seen with any of the PFASs. Activation of peroxisome proliferator-activated receptor-alpha (PPARα) contributes to most of the toxic effects in rodents (Andersen et al., 2008). In addition to PPARα,

activation of nuclear receptors including CAR and PXR in hepatocytes was observed in rodents (Ren et al., 2009; Bjork et al., 2011; Elcombe et al., 2012). A recent study by Beggs, et al. revealed that HNF4α degradation might play a role in PFOA and PFOS induced steatosis and tumorigenesis in human livers (Beggs et al., 2016).

E. Toxicokinetic properties of PFASs in laboratory animals

To date, pharmacokinetic parameters for PFBA (Chang et al., 2008), PFHxA (Chengelis et al., 2009), PFOA (Butenhoff et al., 2004; Hinderliter et al., 2005; Hinderliter et al., 2006a; Hinderliter et al., 2006b; Olsen et al., 2007), PFHpA, PFNA, PFDA, PFBS (Chengelis et al., 2009; Olsen et al., 2009), PFHxS (Olsen et al., 2007; Sundstrom et al., 2012) and PFOS (Olsen et al., 2007; Chang et al., 2012) were studied in at least one species. Both similarities and differences were observed between species, genders as well as among individual PFASs. In terms of similarities, most of the compounds are readily absorbed after oral administration in rats (Chang et al., 2008; Olsen et al., 2009; Sundstrom et al., 2012) with the exception of PFOS (Chang et al., 2012). The other comparatively consistent parameter is the apparent volume of distribution, which is around 200 to 300 ml/kg body weight (corresponding to the extracellular space). Animal studies also demonstrated that PFASs predominantly distribute into plasma and well perfused tissues, such as liver, kidney and lung, and that they are not metabolized in the body because of their extreme stability (Vanden Heuvel et al., 1991). Serum protein binding is another feature for PFASs observed in laboratory

animals and humans (Vanden Heuvel et al., 1991). For example, around 98% of PFOA is bound to rat serum proteins (Hanhijarvi et al., 1982). As a consequence, the renal elimination is limited to the free fraction of PFASs.

F. Renal elimination of PFASs

The major route of elimination for PFASs, especially short-chain PFASs, is through urine in animal models. Since the first observation that renal elimination of PFOA is faster in female than in male rats (Vanden Heuvel et al., 1991), sex-dependent clearance has been reported for various PFASs (Ohmori et al., 2003; Chang et al., 2008; Chengelis et al., 2009; Gannon et al., 2011; Sundstrom et al., 2012). Additionally, when comparing the pharmacokinetic parameters in different species, the elimination half-lives of PFASs differ significantly. Taking the most extensively studied PFOA for example, the elimination half-life ranges from hours to years (1.9 h for female rats, 5.5 h for female rabbits, 7 h for male rabbits, 5.6 d for male mice, 5.6 d for male rats, 15.6 d for female mice, 21.7 d for male mice, 20.9 d for female monkeys, 32.6 d for male monkeys and 3.5 y for humans). Furthermore, a chain length-dependent trend of clearance and elimination half-life of PFCAs was demonstrated in which the total clearance decreases along with the chain length (Ohmori et al., 2003; Han et al., 2012). Although not as dramatic as PFCAs, the clearance of PFSAs shares similar species-, gender- and chain length-dependent pattern. Overall, short-chain PFASs (including PFBA, PFHxA, PFHpA, PFBS, PFHxS) for both sexes and long-chain PFASs (including PFOA and PFNA) for females are readily cleared from the serum in

rats, while the renal clearance for long-chain PFASs (including PFOA and PFNA for males, PFDA and PFOS for both sexes) is limited.

Table 1-3: Summary of elimination half-lives of PFSAs

PFSA	Species	$T_{1/2}$ male (days)	$T_{1/2}$ female (days)	Reference
PFBS (C4+S)	Rats	4.5	3.9	(Olsen et al., 2009)
	Monkeys	95.2	83.2	
	Humans	25.8		
PFHxS (C6+S)	Rats	29.1	1.6	(Sundstrom et al., 2012)
	Mice	30.5	24.8	
	Monkeys	141	87	
	Humans	2665		(Olsen et al., 2007)
PFOS (C8+S)	Rats	38.3	62.3	(Chang et al., 2012)
	Mice	42.8	37.8	
	Monkeys	132	110	
	Humans	1751		(Olsen et al., 2007)

G. Long-chain PFASs accumulate in the liver

Despite more than 90% are bound to plasma protein, the liver-to-plasma ratio of long-chain PFASs is generally higher than 1 in laboratory animals and humans, indicating that long-chain PFASs accumulate in the liver (Seacat et al., 2002; Olsen et al., 2003; Hundley et al., 2006; Kudo et al., 2007; Chang et al., 2012). For instance, the dose recovered from the liver in rats treated with both low-dose and high-dose of PFOA was the highest among all the organs (52% and 27%, respectively). Furthermore, the percentage is considerably higher at the environmentally relevant low dose treatment. However, the only available liver-to-serum ratio of PFOA in humans is around 1 by measurement from pooled human tissues (Maestri et al., 2006). PFOS, on the other hand, was shown to have a higher liver-to-plasma ratio in rats [1.7:1, mean value (Chang et al., 2012)], mice [4:1 with low dose and 1.5:1 with high dose (Bogdanska et al., 2011)], monkeys [1.8: 1, mean value (Seacat et al., 2002)] and humans [1.3: 1, mean value (Olsen et al., 2003)]. The proposed mechanisms for high liver uptake of PFOA and PFOS include their binding to lipid-binding and/or xenobiotic-binding proteins in the liver and being potential substrates for hepatic transporters (Bogdanska et al., 2011).

IV. Concluding remarks for the introduction

Membrane transporter proteins, especially SLC and ABC transporters expressed in the liver, intestine and kidney play important roles in the disposition of xenobiotics. Previous pharmacokinetic analysis for PFASs reveals species and

chain-length differences in their excretion and particularly long half-lives of PFOA, PFHxS and PFOS in humans. Drug transporters likely contribute to these pharmacokinetic characteristics. Identification of which transporters are required for the pharmacokinetic characteristics of PFASs is required to answer these questions: **1) What transporters make it possible for the enterohepatic circulation of PFOA, PFHxS and PFOS; 2) Are transporters responsible for the gender dependent excretion of PFASs; 3) Do transporters play a role in the chain length dependent excretion of PFASs.**

V. Specific aims of this dissertation

The overall goal of my research was to identify and characterize liver, intestinal and kidney transporters that are involved in the liver accumulation and renal clearance of PFCAs and PFSAs in rats and humans. In presenting this dissertation, I will defend two specific aims.

Specific aim 1 evaluates the hypothesis that **liver and intestinal transporters are involved in the disposition of PFASs**. To address this aim, freshly isolated human and rat hepatocytes were used for PFSA uptake measurements. Then, the interactions of PFSAs with uptake transporters expressed in hepatocytes and enterocytes in humans and rats were demonstrated using immortalized cell lines that overexpress individual transporters. Furthermore, the inhibition of efflux transporters expressed at the canalicular membrane of hepatocytes by PFSAs as well as uptake of PFSAs by OSTα-OSTβ expressed at the basolateral membrane of enterocytes were also investigated. The purpose of this study was to identify

the mechanism of the long half-lives of PFOA, PFHxS and PFOS by identifying the liver and intestinal transporters involved in the uptake and efflux of individual PFASs.

Specific aim 2 evaluates the hypothesis that **the differences of renal clearance of PFASs among different species, genders and chain lengths are due to the differences of specific transporters expressed in the kidney**. To address this aim, rat renal transporters OAT1, OAT3 and OATP1A1 were examined for the transport of PFBS, PFHxS and PFOS. The purpose of this study was to identify the renal transporters that contribute to the chain length differences in the excretion of PFSAs.

CHAPTER 2

Materials and methods

I. Materials

Radiolabelled [^{3}H]-taurocholate and [^{3}H]-estrone-3-sulfate were purchased from PerkinElmer (Boston, Massachusetts), potassium perfluorobutane sulfonate (K^+PFBS, 98.2% pure), potassium perfluorohexane sulfonate (K^+PFHxS, >99% pure), and potassium perfluorooctane sulfonate (K^+PFOS, 86.9% pure) were received from the 3M Company (St. Paul, Minnesota). Perfluoroalkyl carboxylates (C7-C10) were purchased from Sigma Aldrich (St Louis, MO).

II. Construction of plasmids

A human NTCP (hNTCP) containing pSport1 vector (Hagenbuch and Meier, 1994) was digested with restriction enzyme *Kpn I* and *BamH I,* and the resulting hNTCP cDNA was inserted into the pcDNA5/FRT vector.

The human ASBT (hASBT) cDNA was sub-cloned from a plasmid purchased from Open Biosystems (OHS6084-202630699, Lafayette, CO) into the pcDNA5/FRT expression vector using PCR and restriction digestion with the primers shown in Table 2-1.

The human OSTα cDNA in pCMV6-XL4 (Origene SC100623, NCBI NM_152672) was digested with *Not*I and inserted into pcDNA5/FRT vector. The human OSTβ

cDNA in pCMV6-Entry (Origene (RC517638, NCBI NM_178859) was sub-cloned into pcDNA5/FRT using primers in Table 2-1.

Rat NTCP (rNTCP) was cloned into the pcDNA5/FRT expression vector from a cell line overexpressing rat NTCP (Schroeder et al., 1998) using the primers as indicated in Table 2-1.

The rat ASBT (rASBT) cDNA was subcloned from a plasmid purchased from Origene (NM_017222, RN209999, Rockville, Maryland) into the pcDNA5/FRT vector using primers and restriction enzymes listed in Table 2-1.

The open reading frames of rOATP1B2 and rOATP2B1 were PCR amplified to include a C-terminal 6 His-tag using the primers containing the restriction sites listed in Table 2-1 from a pSport-1 vector containing the respective cDNAs.

The open reading frames of rOATP1A1 and rOATP1A5 were subcloned using restriction enzymes from a pSport1 vector containing the cDNA of rOATP1A1 or rOATP1A5 into a pExpress1 or a pCMV-pSport6 vector, respectively. Correctness of all sequences was verified by DNA sequencing.

Table 2-1: **Primers used for cloning**

Plasmid	Primers	Restriction site
hASBT	Forward (5'→3'): AGAGAAGCTTCGGGACCATGAATGATCCGAACAGCTG	Hind III
	Reverse (5'→3'): AGAGGGTACCTTATTAATGGTGATGGTGATGATGCTTT TCGTCAGGTTGAAATCC	Kpn I
hOSTβ	Forward (5'→3'): AGAGGCTAGCACCATGGAGCACAGTGAGG	Nhe I
	Reverse (5'→3'): AGAGGCGGCCGCCCTAGCTCTCAGTTTCTGGTACATC	Not I
rNTCP	Forward (5'→3'): AGAGAGCGGCCGCCTAATGGTGATGGTGATGATGATT TGCCATCTGACCAGAATTC	Nhe I
	Reverse (5'→3'): AGAGAGCGGCCGCCTAATGGTGATGGTGATGATGATT TGCCATCTGACCAG	Not I
rASBT	Forward (5'→3'): AGAGGCTAGCACCATGGATAACTCCTCCGTCT	Nhe I
	Reverse (5'→3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGTTT CTCATCTGGTTGA	Not I

rOATP1B2	Forward (5'→3'): AGAGGCTAGCACCATGGACCACACTCAGCAGTCAAG	Nhe I
	Reverse (5'→3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGAAG AGGTGTTTCATTG	Not I
rOATP2B1	Forward (5'→3'): AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGAAG AGGTGTTTCATTG	Kpn I
	Reverse (5'→3'):' AGAGGCGGCCGCCCTAGTGGTGATGGTGATGATGCA GTTC TTGTAGGTCTGAGGCCGCCCTAG	Not I

III. Cell lines and tissue culture

Chinese Hamster Ovary (CHO) Flp-In cells were transfected with the hNTCP-pcDNA5/FRT construct to generate a stable hNTCP expressing cell line.The generation of Chinese Hamster Ovary (CHO) cells stably expressing human OATP1B1 and OATP1B3 as well as the CHO Flp-In cells expressing hOATP2B1 were described previously (Gui et al., 2008; Pacyniak et al., 2010).

CHO Flp-In cells were grown at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM) with 1 g/l D-glucose, 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and 110 mg/l sodium pyruvate supplemented with 10% fetal bovine serum

(FBS) (Hyclone, Logan, Utah), 50 mg/ml L-proline, 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen), and 500 mg/ml hygromycin (Invitrogen, Carlsbad, California).

CHO cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in DMEM with 1 g/l D-glucose, 2 mM L-glutamine, 25 mM HEPES buffer, and 110 mg/l sodium pyruvate supplemented with 10% FBS, 50 mg/ml L-proline, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.5 mg/ml geneticin (not for wild-type cells).

Human embryonic kidney (HEK293) cells (HEK293T/17-CRL-11268, ATCC, Manassas, Virginia) were grown at 37 °C in a humidified 5% CO_2 atmosphere in DMEM High Glucose supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ ml streptomycin.

Human and rat hepatocytes were isolated by the Cell Isolation Core in the Department of Pharmacology, Toxicology and Therapeutics at the University of Kansas Medical Center as described (Xie et al., 2014). All human liver specimens were obtained in accordance with Human Subjects Committee (HSC) at University of Kansas Medical Center approved protocol from patients undergoing hepatic resection procedures or from donor organs. The hepatocytes were then seeded at 250,000 cells per well on collagen-coated 24-well plates and allowed to attach in a humidified 37 °C, 5% CO_2 incubator. Uptake with human hepatocytes was determined 24h after plating and uptake with rat hepatocytes was measured 3 h after plating.

IV. Transporter expression in stable cell lines

For uptake assays, CHO-hNTCP or CHO-hOATP2B1 cells were plated at 40,000 cells per well on 24-well plates and 72h later used for uptake experiments. Medium was changed when needed.

CHO-WT, CHO-OATP1B1, CHO-OATP1B3 cells were plated at 30,000 cells per well on 24-well plates and induced with 5 mM sodium butyrate after 48 hours. Twenty-four hours after induction, cells were used for uptake experiments.

V. Transporter expression in HEK293 cells

HEK293 cells were plated at 20,0000 cells per well in 24-well plates coated with 0.1 mg/ml poly-D-lysine. Twenty-four hours later, cells were transfected with 0.5 mg plasmid DNA and 1.5 ml Fugene HD (Promega, Madison, Wisconsin) per well and uptake assays were performed 48 h later. Medium was changed when needed.

VI. Cell-based transport assay

Cells were washed three times with 1 ml of pre-warmed (37 °C) CHO uptake buffer (116.4 mM NaCl or choline chloride, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM HEPES, pH adjusted to 7.4 with Tris-base), HEK293 uptake buffer (142 mM NaCl or choline chloride, 5 mM KCl, 1

mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.4), or hepatocyte uptake buffer (136 mM NaCl or choline chloride, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, pH 7.4). Then, 200 µl uptake buffer (37 °C) containing PFASs or radiolabeled model substrates were added to the well to initiate transport. Uptake was terminated at indicated time points by two 1-ml washes with ice-cold uptake buffer containing 3% bovine serum albumin and two 1-ml washes with plain ice-cold uptake buffer. Cells were lysed with 200 µl 1% Triton X-100 in H_2O for PFASs or 300 µl 1% Triton X-100 in PBS for radiolabeled substrates at room temperature for 20 min. For PFASs, 120 µl cell lysate was used for liquid chromatography-tandem mass spectrometry (LC-MS/MS). For radiolabeled substrates, 200 µl of cell lysate was transferred to a 24-well scintillation plate (Perkin Billerica, MA) and 750 µl Optiphase Supermix scintillation cocktail (Perkin Elmer, Waltham, MA) was added to each well. Radioactivity was measured in a Microbeta liquid scintillation counter (Perkin Elmer, Waltham, MA). The remaining cell lysates were transferred to 96-well plates to determine the total protein concentration using the bicinchoninic acid protein assay (Pierce Biotechnology, Inc. Rockford, IL). All transport measurements were corrected by the total protein concentration. All experiments were performed two to four times independently with triplicate determinations.

VII. Sf9 vesicle transport

Sf9 vesicles overexpressing human MRP2, BCRP or BSEP were purchased from

Corning Incorporated (Tewksbury, MA). Fifty µg human MRP2, BCRP or BSEP Sf9 vesicles were incubated with model substrates 5(6)-carboxy-2'7'-dichlorfluorescein (CDCF) (Corning gentest MRP/BCRP vesicle assay kit), [^{3}H]-estrone-3-sulfate or [^{3}H]-taurocholate at 37 °C in the presence of 5 mM ATP or AMP for 15 min, 3 min or 30 min, respectively. The reaction was stopped by filtering the vesicle suspension through a 96-well glass fiber filter plate (Millipore, Merck KGaA, Darmstadt, Germany) on a MultiScreenHTS vacuum manifold (Millipore) and the filters were washed 6 times with ice-cold wash buffer from the corresponding vesicle assay kit (Corning). For radiolabeled substrates, Optiphase Supermix scintillation cocktail was added to the filter and it was heat sealed and used directly for counting in a Microbeta liquid scintillation counter (Perkin Elmer). For the fluorescent substrate CDCF, 100 µl 0.1N NaOH was added to each well and the substrate was eluted into a fresh 96-well plate. Fluorescence was determined in a Synergy 2 plate reader (BioTek, Winooski, VT) at 485/525 nm. Transport was determined by subtracting the values in the presence of AMP from the values in the presence of ATP. All experiments were performed two times independently with triplicate determinations.

VIII. LC-MS/MS analyses for PFBS, PFHxS,and PFOS

Cell lysates collected (*vide supra*) were analyzed for PFBS, PFHxS, or PFOS by LC-MS/MS using the following procedures:

(1). Standard and sample preparation

A fixed amount of internal standard (either $^{18}O_2$-PFBS, $^{18}O_2$-PFHxS, or $^{18}O_2$-PFOS) was added to new disposable1.8-ml polypropylene microcentrifuge tubes containing 25 µl of either PFAS standard or lysate samples. PFAS standards were prepared in the same buffer matrix as lysate samples.

To all tubes, 175 µl of a solution containing 5% ammonium acetate (2 mM) and 95% acetonitrile was added followed by vortex (approximately 5 seconds) and then centrifugation (2500 *x g*, 20 minutes, room temperature). Subsequently, 180 µl of the supernatant was aliquoted to a new 100 µl polypropylene auto sampler vial pending LC-MS/MS analyses.The reagent-grade water used for all the LC-MS/MS analyses was MilliQ deionized water that was further purified to remove residual traces of perfluorinated compounds by being pumped through a C-18 HPLC column prior to use.

(2). LC-MS/MS Conditions

The instrument used for analysis was an API 5000 mass spectrometer (Applied Biosystems / MDS-Sciex Instrument Corporation, Foster city, CA) configured with Turbo Ion Spray (pneumatically assisted electrospray ionization source) in negative ion mode. Separation of the compounds was completed on a Mac-Mod ACE ® C-18, 5 µm, 75 x 2.1 mm i.d. HPLC column with a gradient flow rate of 0.250 ml/min (dual column method) using the conditions specified in Table 2-2.

All source parameters were optimized under these conditions according to manufacturer's guidelines. Transition ions monitored were as follows:

PFBS: 299 -> 80 amu; PFBS Internal Standard: dual $^{18}O_2$-labeled PFBS: 303 -> 84 amu; PFHxS: 399 -> 80 amu; PFHxS Internal Standard: triple $^{18}O_2$-labeled PFHxS: 405 -> 86 amu; PFOS: 499 -> 80 amu; PFOS Internal Standard: dual $^{18}O_2$-labeled PFOS: 503 -> 84 amu.

Table 2-2: HPLC separation conditions of PFBS, PFHxS,and PFOS

The gradient was run with mobile phase A (2 mM ammonium acetate) and mobile phase B (acetonitrile).

Time (min)	Mobile Phase B %
0.01	30
1.80	30
3.50	60
4.50	60
5.50	90
7.00	90
8.00	30
11.50	30
11.55	End of run

IX. Statistical analysis

Data were analyzed for significant differences using one-way ANOVA followed by the Dunnett post-test for inhibition assays and Student's t-test for one time point uptake determinations. $p<0.05$ was considered significant.

CHAPTER 3

Na^+/taurocholate cotransporting polypeptide (NTCP) and apical sodium dependent bile acid transporter (ASBT) are involved in the disposition of PFSAs in humans and rats

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I. Introduction

Pefluoroalkyl sulfonates (PFSAs) are fluorinated fatty acid analogues used as surfactants in industrial and commercial applications (Kissa and Kissa, 2001; Buck et al., 2011). Due to the unique physicochemical properties of the carbon-fluorine bonds, certain PFSAs such as perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonate (PFOS) are resistant to environmental and biological degradation. They are frequently detected in the environment (Calafat et al., 2007; Fromme et al., 2009; Houde et al., 2011; Kato et al., 2011; Zhao et al., 2012). Consequently, PFOS has been nominated to the Stockholm Convention in 2009 as a persistent organic pollutant (Convention, 2009).

Pharmacokinetic studies revealed that PFSAs primarily bind to serum proteins and that their clearance is species- and chain length-dependent (Andersen et al., 2008). In Sprague Dawley rats, the estimated serum elimination half-life for PFOS (an eight-carbon homolog) is approximately one month (Chang et al., 2012) whereas PFBS (a four-carbon homolog) is efficiently excreted in urine with an estimated serum half-life of 3.9-4.5 hours (Olsen et al., 2009). In these rats,

there is a distinct gender-difference in the serum elimination of PFHxS (a six-carbon homolog) in that the estimated half-lives are 30 days in male but only 2 days in female rats (Sundstrom et al., 2012). In cynomolgus monkeys, the estimated serum elimination half-lives for PFHxS and PFOS are approximately four months (Chang et al., 2012; Sundstrom et al., 2012); whereas, for PFBS the respective half-life is approximately 4 days (Olsen et al., 2009). The estimated serum elimination half-lives of PFHxS and PFOS in human serum are several years (geometric means are 7.3 years [95% CI 5.8 – 9.2 years] and 4.8 years [95% CI 4.0 – 5.8 years], respectively) (Olsen et al., 2007). In contrast, PFBS has an estimated geometric serum elimination half–life of 26 days (95% CI 16 – 40 days) (Olsen et al., 2009).

Several studies have demonstrated that PFOS preferentially accumulates in the liver. In Sprague Dawley rats given a single IV dose of ^{14}C-radiolabelled PFOS, Chang *et al.* (2012) reported that 3% and 25% of the administered dose was recovered in plasma and liver, respectively, after 89 days. They also reported data for CD-1 mice and Sprague Dawley rats given single oral doses. The data demonstrated that liver PFOS concentrations were always higher than concurrent serum PFOS concentrations by a factor of approximately 2 to 3 at measured time points following dosing. This is consistent with observations from repeat-dose studies in Sprague Dawley rats where the liver-to-serum PFOS concentration ratios ranged from 2.5 – 12.2 in Sprague Dawley rats after 4 to 14

weeks of dosing (Seacat et al., 2002). Thus, these data suggest that PFOS is preferentially distributed to the liver and may undergo enterohepatic circulation.

There is some evidence that enterohepatic circulation may contribute to the preferential distribution of PFSAs to liver. Male rats were given a single IV dose of ^{14}C-labelled PFOS and followed for 21 days during which they were given either a basal diet or a diet containing the bile acid sequestrant, cholestyramine (Johnson et al., 1984). Fecal elimination of PFOS was increased approximately 10-fold, and liver and serum PFOS concentration were reduced by 75% and 85%, respectively, in rats given cholestyramine in their diet as compared to rats fed basal diet. In humans, a case study involving a single human subject provided evidence that cholestyramine was effective in removing both PFOS and PFHxS via fecal elimination (Genuis et al., 2010).

Although previous studies have demonstrated that certain perfluoroalkyl carboxylates are substrates of members of the organic anion transporting polypeptide (OATP) and the organic anion transporter (OAT) family (Nakagawa et al., 2008; Weaver et al., 2010; Yang et al., 2010; Han et al., 2012), interactions between PFSAs and transporters might not be limited to OATPs and OATs. During the process of enterohepatic circulation of bile salts, it is well-known that the two uptake transporters Na^+/taurocholate cotransporting polypeptide (NTCP) and apical sodium-dependent bile salt transporter (ASBT) play important roles (Hagenbuch and Dawson, 2004). NTCP is highly expressed at the basolateral

membrane of hepatocytes and mediates the uptake of bile acids into hepatocytes in a sodium-dependent manner (Claro da Silva et al., 2013). Beside bile acids, other known substrates of NTCP include steroid sulfates such as estrone-3-sulfate (Schroeder et al., 1998), anti-hyperlipidemic drugs like rosuvastatin (Ho et al., 2006), and drug conjugates such as chlorambucil-taurocholate (Kullak-Ublick et al., 1997). When bile acids reach the gastrointestinal tract, ASBT localized to the brush-border membrane in the terminal ileum reabsorbs the majority of them and OSTα/β exports them across the basolateral membrane of enterocytes (Ballatori et al., 2005). ASBT is also expressed at the apical membrane of renal proximal tubular epithelial cells and at the apical membrane of cholangiocytes. Similar to NTCP, the transport by ASBT depends on the sodium gradient across the cell membrane. In contrast to NTCP, ASBT has narrow substrate specificity, which is restricted to bile acids (Claro da Silva et al., 2013).

Given the evidence that PFOS and PFHxS might undergo enterohepatic circulation and given the important role the bile salt transporters play in the enterohepatic circulation, we hypothesized that bile salt transporters are involved in the disposition of PFSAs. In this study, we investigated the roles of human and rat NTCP and ASBT as well as of human OSTα/β in transporting PFBS, PFHxS, and PFOS. Initial inhibition studies and uptake experiments indicated the potential of the interactions between PFSAs and the transporters. Based on these results, additional time dependency and kinetic studies were performed.

II. Results

2.1 Uptake of PFSAs by human and rat hepatocytes

To determine whether or not sodium-dependent transporters are involved in the uptake of PFSAs into hepatocytes, uptake of 50 μM PFBS, PFHxS, and PFOS were measured at 2 min using freshly isolated human and rat hepatocytes. First, sodium-dependent uptake of [^{3}H]-taurocholate was used to ascertain that the isolated hepatocytes were functional (Fig. 3-1). Then, sodium-dependent uptake of the three PFSAs were measured in the same batches of hepatocytes. As shown in Figure 3-2A, sodium-dependent uptake was observed for all three PFSAs in human hepatocytes. Under the experimental conditions used, the sodium-dependent uptake (net uptake, black bar) was lowest for PFBS while the signal for PFHxS and PFOS was similar and larger. Sodium-dependent uptake also was observed in rat hepatocytes for PFBS, PFHxS and PFOS (Fig. 3-2B). Uptake was also measured in the presence of 100 μM bromosulfophthalein, a known inhibitor of both NTCP and the OATPs. BSP inhibited both sodium-dependent and sodium-independent uptake of PFBS and to a lesser degree also PFHxS, while only the sodium-dependent portion of PFOS was inhibited (Fig. 3-2).

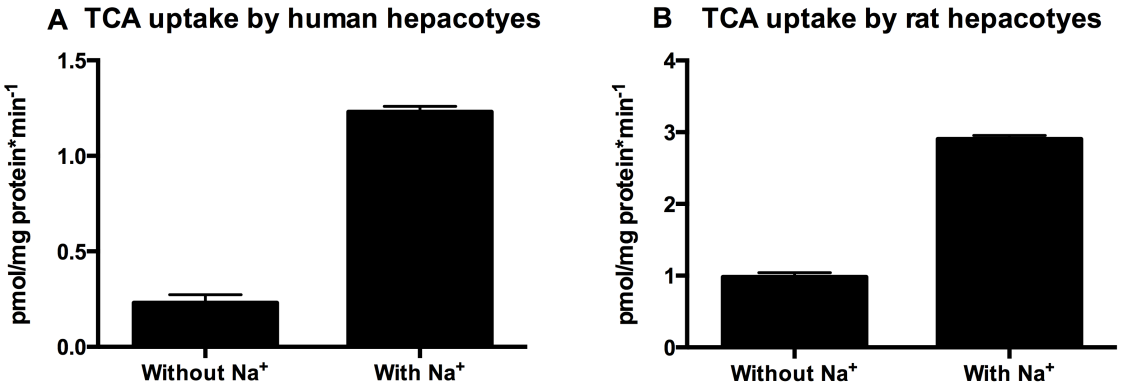


Figure 3-1: TCA uptake by hepatocytes

Taurocholate uptake mediated by human and rat hepatocytes. Uptake of 30 nM [^{3}H]-TCA was measured with freshly isolated human (A) or rat (B) hepatocytes in the absence or presence of sodium for 1 min at 37°C. Each bar represents the mean ± SD of the uptake of triplicates. The results were corrected for total protein concentration in each well.

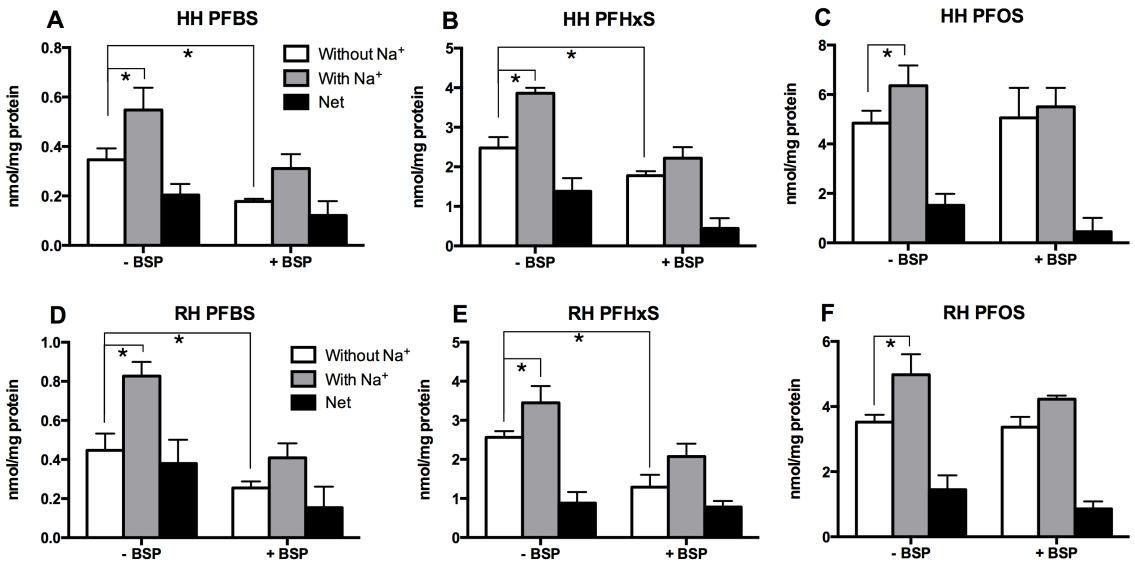


Figure 3-2: PFSA uptake into freshly isolated human (A-C) and rat (D-F) hepatocytes

Uptakes of 50 μM PFBS, PFHxS, and PFOS in the absence or presence of 100 μM bromosulfophthalein (BSP) was measured in freshly isolated human (HH) and rat hepatocytes (RH) at 2 min in the absence (white bars) or presence (gray bars) of sodium. Net sodium-dependent uptake (black bars) was calculated by subtracting the value of uptake in the absence of sodium from uptake in the presence of sodium. Each bar represents the mean ± SD from three independent experiments with triplicate determinations. The results were corrected for total protein concentration in each well. *p<0.05.

2.2 Uptake of PFSAs by human and rat NTCP

To test whether PFSAs would interact with human or rat NTCP, uptake of the model substrate [^{3}H]-taurocholate was measured in the absence or presence of 10 μM PFBS, PFHxS or PFOS. In CHO Flp-in cells stably expressing human NTCP, this uptake was inhibited in a chain length-dependent way with PFOS inhibiting the strongest, followed by PFHxS and then PFBS (Fig. 3-3A). In HEK293 cells transiently expressing rat NTCP, PFHxS showed the strongest inhibition of [^{3}H]-taurocholate uptake (50% inhibition) while PFBS and PFOS inhibited only by 20 to 25% (Fig. 3-3B).

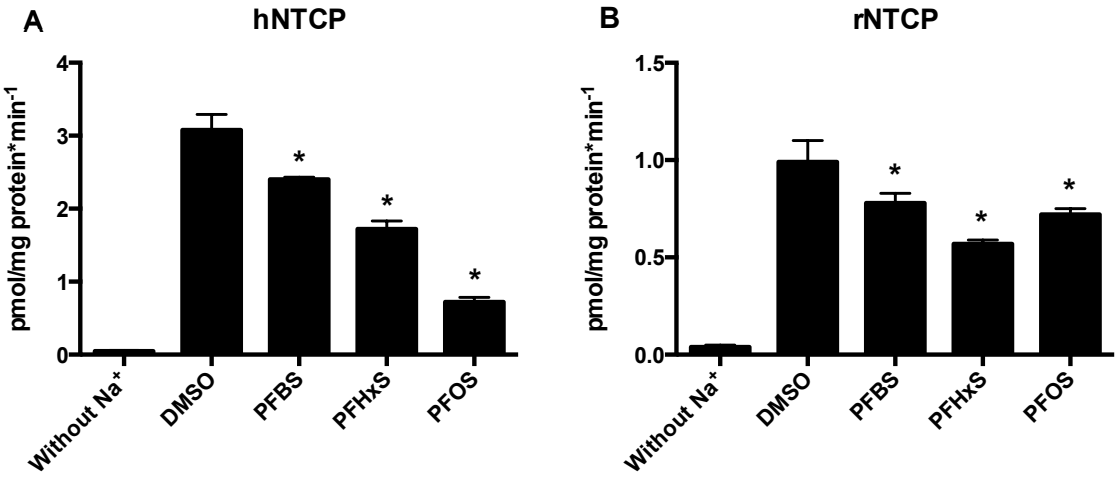


Figure 3-3: Inhibition of ^{3}H-taurocholate uptake mediated by human (A, hNTCP) and rat (B, rNTCP) NTCP by PFSAs

Human NTCP-mediated 30 nM [^{3}H]-taurocholate uptake was measured at 37°C for 1 min in the absence (DMSO) or presence of 10 μM PFBS, PFHxS, and PFOS using the CHO-hNTCP cell line. Rat NTCP-mediated 30 nM [^{3}H]-taurocholate uptake was measured at 37°C for 1 min in the absence or presence of 10 μM PFBS, PFHxS, and PFOS using HEK293 cells transiently transfected with rNTCP. Each bar represents the mean ± SD of triplicate determinations. The results were corrected for total protein concentration in each well. *p<0.05 compared to DMSO control.

Based on the results of the inhibition experiments that demonstrated interactions with all three PFSAs, direct uptakes of PFBS, PFHxS, and PFOS by human and rat NTCP were determined and quantified by LC-MS/MS. As shown in Figure 3-4A, at 10 μM substrate and 1 min incubation, sodium-dependent uptake by human NTCP was highest for PFOS, followed by PFHxS and PFBS mirroring the chain length-dependent inhibition seen with PFCAs. In contrast, uptake by rat NTCP was similar for all three PFSAs at the given experimental condition (Fig. 3-4B).

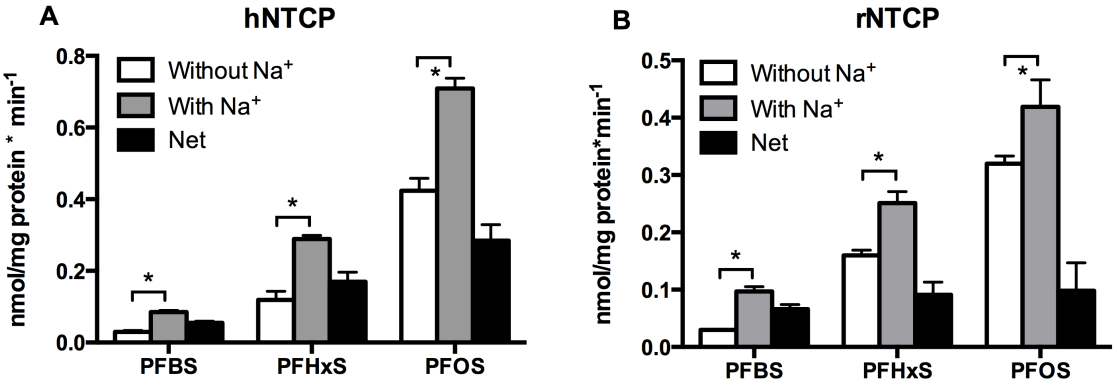


Figure 3-4: Uptake of PFSA by human (A, hNTCP) and rat (B, rNTCP) NTCP

CHO-hNTCP cells or HEK293 cells transiently transfected with rNTCP were used to measure the uptake of 10 μM PFBS, PFHxS, and PFOS in the absence (white bars) and presence (gray bars) of sodium. Net sodium-dependent uptake (black bars) was calculated by subtracting the values of uptake in the absence of sodium from uptake in the presence of sodium. Each bar represents the mean ± SD from two independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. *p<0.05.

In order to further characterize the transport by NTCP, uptakes of PFBS, PFHxS and PFOS were measured in a time dependent manner at a low (10 μM) as well as at a high substrate concentration (200 μM for PFBS and 400 μM for PFHxS and PFOS). At the low concentration, the initial linear portion of human NTCP-mediated transport was different between the three PFSAs (Fig. 3-5A-C) and extended up to 60 seconds for PFOS. For rat NTCP the initial linear range was between 20 seconds for PFBS (Fig. 3-5D) and 30 seconds for both PFHxS (Fig. 3-5E) and PFOS (Fig. 3-5F). At the high substrate concentration, uptake of all PFSAs by human and rat NTCP was linear up to at least 20 seconds (data not shown).

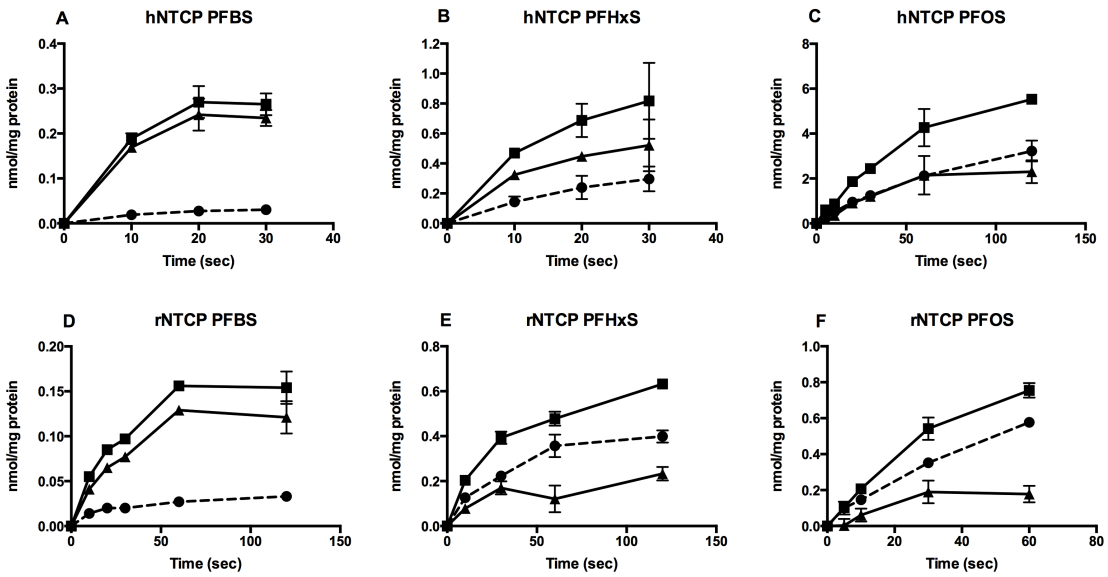


Figure 3-5: Time dependent uptake of PFSAs by human (A, B, C; hNTCP) and rat (D, E, F; rNTCP) NTCP

Uptake of 10 μM PFBS (A, D), PFHxS (B, E), and PFOS (C, F) was measured at 37°C at the indicated time points using CHO-hNTCP cells and HEK293 cells transiently transfected with rNTCP in the absence (circles) and presence (squares) of sodium. Net sodium-dependent uptake (triangles) was calculated by subtracting the value of uptake in the absence of sodium from uptake in the presence of sodium. The results were corrected for total protein concentration in each well. Each point represents the mean ± SD of triplicates.

Based on the results from the time-dependent uptake experiments, all concentration-dependent transport measurements for kinetic analysis of PFBS, PFHxS and PFOS were determined at 10 seconds. Concentration-dependent net sodium-dependent uptake of PFBS, PFHxS and PFOS by human NTCP, and PFBS and PFHxS by rat NTCP is shown in Figure 3-6. Kinetic parameters were calculated based on the Michaelis-Menten equation and the resulting K_m and V_{max} values are summarized in Table 1. PFBS was transported by human NTCP with the highest affinity (K_m value of 39.6 μM) but the lowest V_{max} value while both PFHxS (K_m = 112 μM) and PFOS (K_m = 130 μM) had lower affinities and higher maximal transport rates. The intrinsic clearance (V_{max}/K_m) was similarly twice as high for the two substrates with the higher V_{max} values. With respect to rat NTCP, although PFBS was transported with a higher affinity (K_m =76.2 μM) than PFHxS (K_m=294 μM) the difference in the V_{max} values resulted in equal intrinsic clearances for both compounds (Table 3-1). The kinetic parameters of PFOS by rat NTCP was not determined due to low signal-to-noise ratio of the transport.

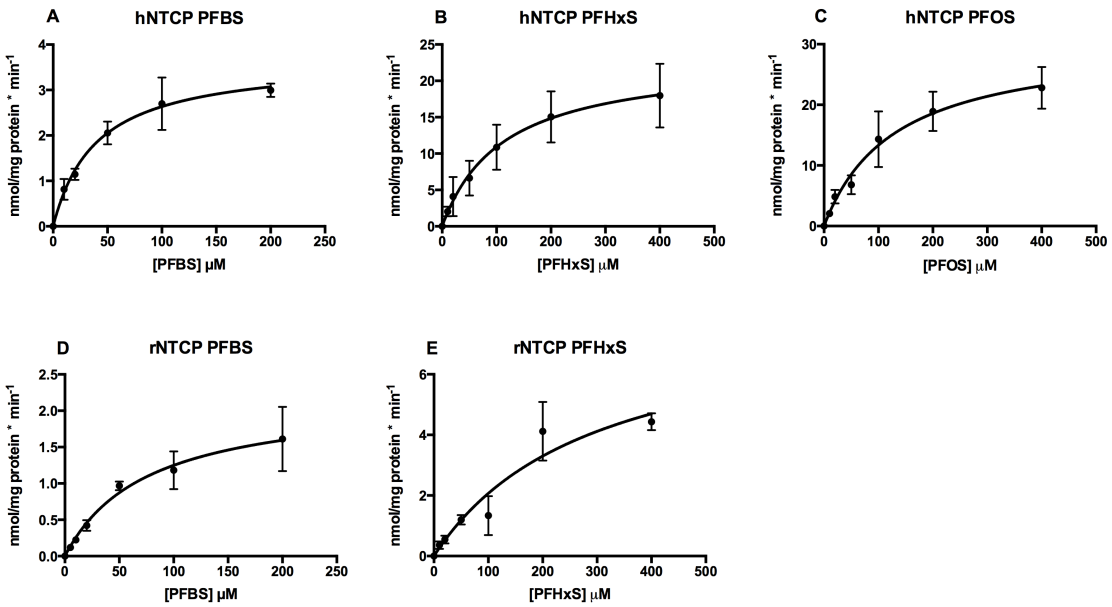


Figure 3-6: Kinetics of human (A, B, C) and rat (D, E) NTCP-mediated transport of PFBS (A, D), PFHxS (B, E), and PFOS (C)

Uptake of increasing concentrations of PFBS, PFHxS, and PFOS was measured within the initial linear range of transport using CHO-hNTCP cells (A, B, C) and HEK293 cells transiently transfected with rNTCP (D, E). Net uptake was calculated by subtracting the values of uptake in the absence of sodium from uptake in the presence of sodium and was corrected for total protein concentration. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 3-1). Each point represents the mean ± SD from three to four independent experiments performed in triplicates.

Table 3-1: Kinetic parameters of PFBS, PFHxS and PFOS transport mediated by human or rat NTCP

Transporter	PFSA	K_m(μM)	V_{max} (nmol/mg protein*min^{-1})	V_{max}/K_m(ml/mg protein*min-^{1})
hNTCP	PFBS	39.6±8.2	3.7±0.3	0.1±0.02
	PFHxS	112±32.9	23.2±3.0	0.2±0.07
	PFOS	130 ±32.9	30.7±3.2	0.2±0.06
rNTCP	PFBS	76.2±23.6	2.2±0.3	0.03±0.01
	PFHxS	294±121	8.1±1.8	0.03±0.01

2.3 Inhibition of human MRP2, BCRP and BSEP transport by PFSAs

To determine whether PFSAs interact with efflux transporters in the hepatocytes, the transport of model substrates by human MRP2, BCRP and BSEP in Sf9 vesicles was measured in the absence or presence of 10 μM or 100 μM PFBS, PFHxS or PFOS. Transport of CDCF by human MRP2 was not affected by PFBS (Fig. 3-7A). At 10 μM, only PFOS inhibited CDCF transport, while at 100 μM, both PFHxS and PFOS showed inhibition, with PFOS inhibiting transport by over 80% (Fig. 3-7A). Transport of [^{3}H]-estrone-3-sulfate mediated by human BCRP was decreased in the presence of all three PFSAs, however, only the inhibition by 100 μM PFHxS was significant (Fig. 3-7B). In addition, BSEP-mediated transport of [^{3}H]-taurocholate was only inhibited by 100 μM PFOS but not by any of the other conditions (Fig. 3-7C).

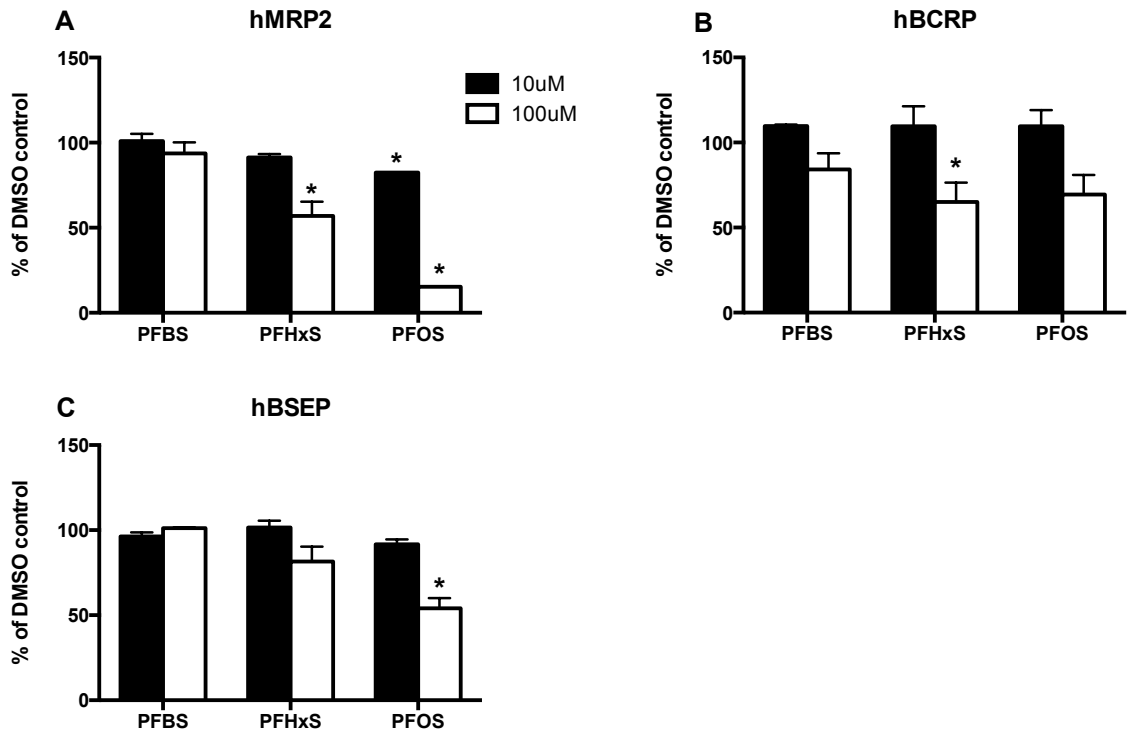


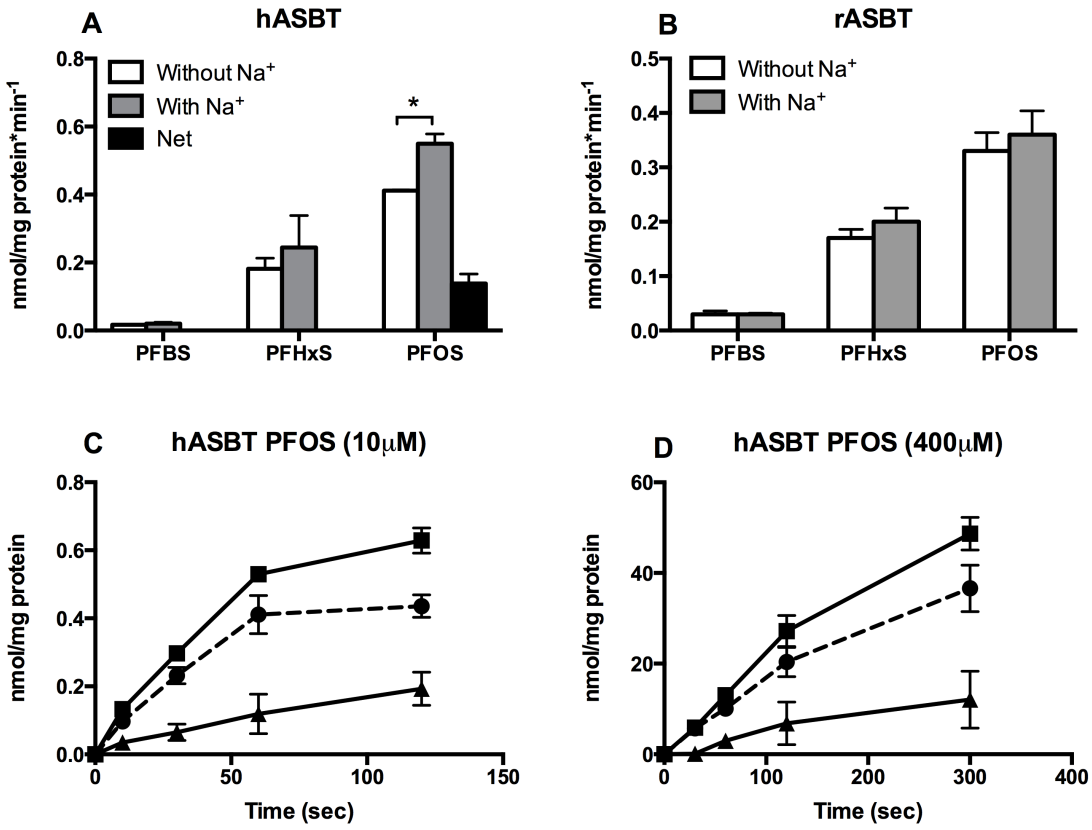
Figure 3-7: Inhibition of human MRP2 (A), BCRP (B) and BSEP (C) transport by PFSAs

Transport of 5 μM CDCF, 0.01 μM [^{3}H]-estrone-3-sulfate or 0.5 μM [^{3}H]-taurocholate by human MRP2, BCRP and BSEP vesicles were measured with or without 10 μM (Black bar) or 100 μM (white bar) PFBS, PFHxS and PFOS. Each bar represents the mean ± SD of triplicate determinations. *$p<0.05$ compared to DMSO control.

2.4 Transport of PFSAs by human and rat ASBT

ASBT is another sodium-dependent transporter belonging to the same gene family as NTCP. It is normally expressed at the apical membrane of ileal enterocytes, cholangiocytes and renal proximal tubular epithelial cells and transports bile acids in a sodium-dependent way. In HEK293 cells transiently expressing human or rat ASBT, uptake of 10 μM PFBS, PFHxS or PFOS was measured at 1 minute in the absence or presence of sodium. Sodium-dependent uptake was only observed for PFOS by human ASBT (Fig. 3-8A) while none of the three PFSAs were transported by rat ASBT (Fig. 3-8B). Human and rat ASBT function was confirmed by measuring sodium-dependent transport of the model substrate [^{3}H]-taurocholate (Fig. 3-9).

Time-dependent uptake was performed for human ASBT-mediated PFOS in order to further characterize its kinetics. Uptake was linear up to 2 minutes at both the low (10 μM) and high (400 μM) PFOS concentration (Fig. 3-8C and Fig. 3-8D). However, the kinetic parameters of PFOS transport could not be determined with confidence due to a low signal-to-noise ratio.


A
hASBT
Without Na+
With Na+
Net
*
nmol/mg protein*min-1
0.8
0.6
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0.0
PFBS
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PFOS
B
rASBT
Without Na+
With Na+
nmol/mg protein*min-1
0.5
0.4
0.3
0.2
0.1
0.0
PFBS
PFHxS
PFOS
C
hASBT PFOS (10μM)
nmol/mg protein
0.8
0.6
0.4
0.2
0.0
0
50
100
150
Time (sec)
D
hASBT PFOS (400μM)
nmol/mg protein
60
40
20
0
0
100
200
300
400
Time (sec)

Figure 3-8: Uptake of PFSAs by human (A, C, D) and rat (B) ASBT

HEK293 cells transiently transfected with hASBT (A) or rASBT (B) were used to measure uptake of 10 μM PFBS, PFHxS, and PFOS in the absence (white bars) or presence (gray bars) of sodium for 2 minutes. Net sodium-dependent uptake (black bars) was calculated by subtracting the values of uptake in the absence of sodium from uptake in the presence of sodium. Each bar represents the mean ± SD of triplicates from two independent experiments. (C, D) Uptake of 10 μM PFOS was measured at 37°C for the indicated time points by HEK293 cells transiently transfected with hASBT in the absence (circles) or presence (squares) of sodium. Net sodium-dependent uptake (triangles) was calculated by subtracting the values of uptake in the absence of sodium from uptake in the presence of sodium. The results were corrected for total protein concentration in each well. Each point represents the mean ± SD of triplicates. *p<0.05.

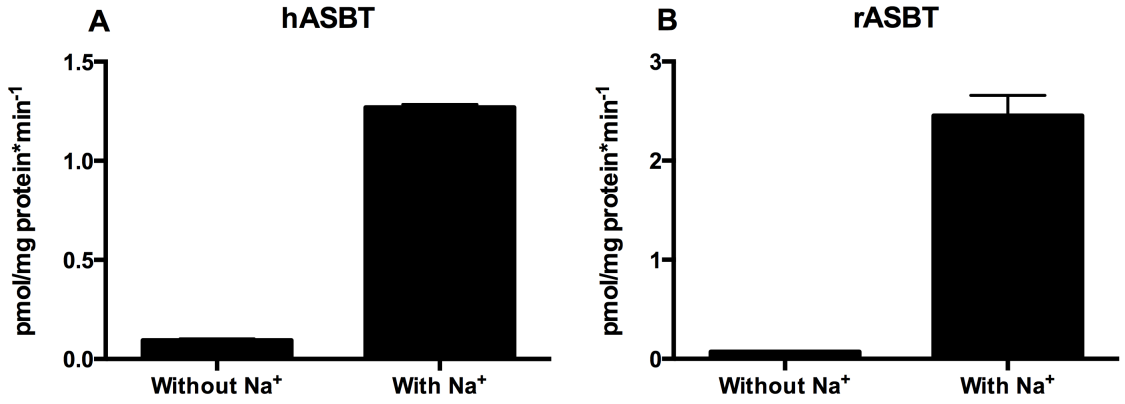

Figure 3-9: Taurocholate uptake mediated by human and rat ASBT

Using HEK293 cells transiently transfected with human (A) or rat (B) ASBT cDNA, uptake of 30 nM [^{3}H]-TCA was measured in the absence or presence of sodium for 1 min at 37°C. Each bar represents the mean ± SD of the uptake of triplicates. The results were corrected for total protein concentration in each well.

2.5 Transport of PFSAs by human OSTα/β

Human OSTα/β is expressed at the basolateral membrane of enterocytes to mediate the efflux of bile acids. Since it can transport bi-directionally depending on the concentration gradient of substrates, uptake of 10 μM PFSAs was measured at 2 and 10 min after establishing the proper function of OSTα/β (Fig. 3-10). At both time points, uptake of all three PFSAs was higher in human OSTα/β transfected HEK293 cells than in empty vector transfected cells and net uptake was higher at 10 min as compare to 2 min (Fig. 3-11).

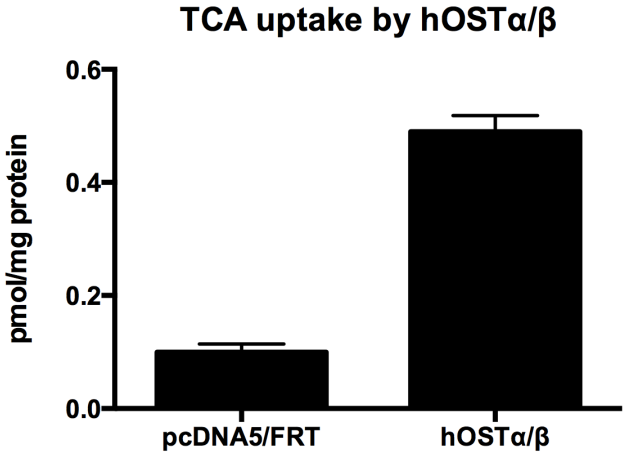

Figure 3-10: Human OSTα/β-mediated transport of TCA

Using HEK293 cells transiently transfected with human OSTα and OSTβ cDNA containing plasmid, uptake of 30 nM [^{3}H]-TCA was measured for 2 min at 37°C. Each bar represents the mean ± SD of the uptake of triplicates. The results were corrected for total protein concentration in each well.

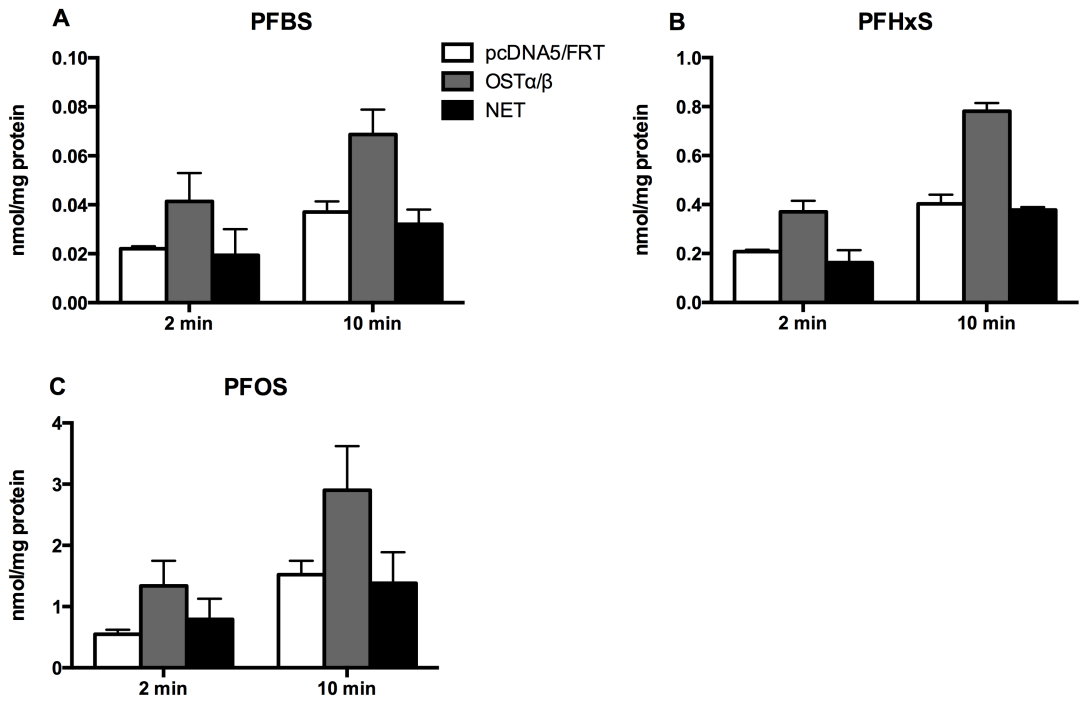

Figure 3-11: Uptake of PFSAs by human OSTα/β

HEK293 cells transiently transfected with hOSTα/β (gray bars) or empty vector pcDNA5/FRT (white bars) were used to measure uptake of 10 μM PFBS (A), PFHxS (B) or PFOS (C) at 2 min and 10 min. Net uptake (black bars) was calculated by subtracting the values of uptake in empty vector transfected cells from uptake in the hOSTα/β transfected cells. The results were corrected for total protein concentration in each well. Each bar represents the mean ± SD of triplicates from three independent experiments.

III. Discussion

In this chapter, we have demonstrated that uptake of PFSAs into human and rat hepatocytes is mediated by sodium-dependent and sodium-independent mechanisms. Furthermore, we showed that all three PFSAs, PFBS, PFHxS and PFOS, are substrates for human and rat NTCP. Carrier-mediated uptake by rat hepatocytes was reported previously for PFOA and bromosulphophthalein (BSP), an inhibitor of OATPs and NTCP, can inhibit this transport (Han et al., 2008). However, specific transporters were not identified until now. In addition to NTCP, we also demonstrated that human ASBT can mediate the uptake of PFOS. To the best of our knowledge, this is the first time that sodium-dependent transport is reported for PFASs. Furthermore, human OSTα/β was able to transport all three PFSAs.

We used BSP inhibition to get an idea whether the sodium-independent OATPs or the sodium-dependent NTCP would be the major transport system for the uptake of PFSAs into hepatocytes. The fact that sodium-independent uptake of PFOS was hardly inhibited by BSP (Fig. 3-2) suggests that NTCP is the major uptake transporter and that OATPs, at most, play a minor role. The shorter chain PFBS and PFHxS are probably transported by both types of transporters but again, NTCP seems to be the major carrier given the minor inhibition of the sodium-independent uptake (Fig. 3-2).

Fenestration of sinusoids in combination with specific transporters in the sinusoidal membrane of hepatocytes allows excretion of numerous protein bound xenobiotics by the liver. These fenestrae allow the protein-bound compounds to get into close proximity with the uptake transporters in hepatocytes while protein binding prevents their glomerular filtration in the kidneys. Both PFOS and PFHxS are highly protein-bound in the plasma, predominately to albumin (Kerstner-Wood, 2004; Butenhoff et al., 2012), and given that NTCP can transport these PFSAs and is abundantly expressed in the sinusoidal membrane, could be the major mechanism for the previously reported preferential liver accumulation of these compounds (Maestri et al., 2006; Karrman et al., 2010).

In humans, biliary excretion has been suggested as the main route of elimination for PFOS and has been estimated to be 200-fold higher than urinary excretion (Harada et al., 2007). The estimated reabsorption rates are 97 percent and 95 percent in humans and rats, respectively, very comparable between the two species (Harada et al., 2007). Our findings that in particular PFOS but to a lesser degree also PFHxS were able to inhibit MRP2, BCRP and BSEP, three of the ABC transporters expressed in the canalicular membrane of human hepatocytes, suggests that these transporters are likely candidates for canalicular secretion of PFOS and PFHxS. We would like to emphasize that inhibition does not necessarily mean that the inhibitors are substrates of the transporters they inhibit and as a consequence these three transporters should be tested in the future whether they directly mediate transport of PFOS and PFHxS.

We demonstrated that human ASBT can transport PFOS when expressed in HEK293 cells. Assuming that human ASBT can transport PFOS also *in vivo* would suggest that PFOS is absorbed in the intestine preferentially compared to other PFSAs. The relative high unspecific background seen in our data indicates that passive diffusion of PFOS probably plays an important role as well. We had preliminary data that additional transporters expressed in the small intestine like human OATP2B1 (Drozdzik et al., 2014) and rat OATP1A5 (Walters et al., 2000b) could play a role in the reabsorption of PFSAs, especially in the case of PFHxS and PFBS which were not transported by rat ASBT. The detailed functional characterization of these transporters will need to be presented in the following chapter of this dissertation.

In addition to enterocytes, ASBT is also expressed in the epithelial cells lining the bile duct where it samples biliary contents for signaling to the hepatocytes and is part of the cholehepatic shunt pathway (Benedetti et al., 1997; Meier and Stieger, 2002; Xia et al., 2006). Due to the transport by ASBT, cholehepatic shunting of PFOS might be an additional critical component for its retention in human liver. Serum albumin bound PFOS likely limits its glomerular filtration (Butenhoff et al., 2012). In addition, ASBT present in the brush border membrane of proximal tubular epithelial cells (Hagenbuch and Dawson, 2004) could reabsorb the limited amount of unbound PFOS filtered, keeping the urinary excretion at its very low levels.

Although we showed that PFBS is a substrate of both human and rat NTCP, the human serum elimination half-life has been estimated to be around 26 days and urine was shown to be the dominant route of elimination (Olsen et al., 2009). This significant urinary excretion could be due to glomerular filtration or due to transporters of the OAT family that are expressed in the kidney (OAT1 and OAT3). Because these carriers are involved in the transport of the shorter chain PFCAs (Weaver et al., 2010) they are also likely candidates for PFBS secretion (see Chapter 5).

It is well accepted that human NTCP transports, in addition to bile acids, other endo- and xenobiotics, including some of the statins (Bi *et al.*, 2013). Even though ASBT is a bile acid cotransporter closely related to NTCP, to date, known substrates of ASBT have been restricted to bile acids (Hagenbuch and Dawson, 2004; Dawson, 2011). Thus, the identification of PFOS as an ASBT substrate represents a very novel finding. This raises the possibility that other compounds similar to PFOS might be substrates of ASBT.

In conclusion, our studies reveal that PFBS, PFHxS, and PFOS are substrates for the sodium-dependent liver transporter NTCP in humans and rats, and that human ASBT transports PFOS. The three ABC transporters MRP2, BCRP and BSEP are inhibited in particular by PFOS, and human OSTα/β can transport all three PFSAs. Thus, the combined action of the hepatic NTCP and ABC transporters, together with the intestinal ASBT and OSTα/β likely facilitates the

enterohepatic circulation of PFHxS and PFOS. In addition, ASBT expressed in cholangiocytes possibly contributes to cholehepatic shunting of PFOS. Both pathways together enhance liver accumulation and likely contribute to the long serum elimination half-life seen for PFOS in humans.

CHAPTER 4

Organic anion transporting polypeptides contribute to the disposition of perfluoroalkyl acids in humans and rats

I. Introduction

Perfluoroalkyl acids (PFAAs), including perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs), are fluorinated fatty acid-like chemicals, which are widely used in commerce since the 1950s (Prevedouros et al., 2006; Buck et al., 2011). Certain PFAAs have received attention in recent years, because they are resistant to environmental degradation and frequently detectable in human blood (Fromme et al., 2009; Kato et al., 2011; Zhao et al., 2012). The presence of certain PFAAs in human blood may, in part, be due to their retention in the body through renal proximal tubule reabsorption and enterohepatic circulation after exposures from various environmental sources (Andersen et al., 2008).

There are several differences in the elimination kinetics of PFAAs depending on species, genders within species, and number of carbons in the perfluoroalkyl chain. In particular, the PFSAs, perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) as well as the PFCA, perfluorooctanoate (PFOA), have serum elimination half-lives in humans of several years (Olsen et al., 2007; Bartell et al., 2010). Pharmacokinetic studies on these three PFAAs in non-humans have demonstrated considerable variation in serum elimination half-lives between species (Hundley et al., 2006; Chang et al., 2012; Sundstrom et al.,

2012) and, in some cases, between genders within species (Hundley et al., 2006; Sundstrom et al., 2012), but are generally much shorter than the reported human serum elimination half-lives. For example, for PFOS, the reported serum elimination half-lives in rats and mice were on the order of 1–2 months and, in monkeys, approximated 4 months (Chang et al., 2012), as opposed to several years in humans (Olsen et al., 2007). In another example, the serum elimination half-life of PFOA in female rats is a few hours versus several days in male rats (Kennedy et al., 2004; Hundley et al., 2006).

In addition to these species and gender differences in elimination for certain PFAAs, PFAAs with fewer carbons in the perfluoroalkyl chain tend to have shorter serum elimination half-lives than their longer homologs (Lau, 2015). For example, the serum elimination half-lives in humans for perfluorobutane sulfonate (PFBS) and perfluorohexanoate (PFHxA) are approximately one month (Olsen et al., 2009; Russell et al., 2013) versus several years for PFOS and PFOA (Olsen et al., 2007; Bartell et al., 2010).

In attempting to explain these kinetic differences, attention has focused on the role of membrane transporters. Kudo et al. (Kudo et al., 2002) first provided experimental support for sex hormone-mediated expression of organic anion transporters (OATs) in rat kidneys to explain male and female differences in clearance of PFOA, and suggested a possible role for organic anion transporting polypeptide 1A1 (OATP1A1) in proximal tubule reabsorption of PFOA in male

rats. Subsequent experimental and modelling studies have provided substantial additional support for role of transporter-mediated renal proximal tubule reabsorption in the differential retention of PFOA (Han et al., 2003; Andersen et al., 2006; Katakura et al., 2007a; Yang et al., 2009; Weaver et al., 2010; Loccisano et al., 2011). At this point in time, it is fair to conclude that renal transporters play a key role in defining the kinetic characteristics of PFAAs.

While there has been a fair amount of effort to investigate renal proximal tubule transporters, fewer studies have focused on the role of enterohepatic circulation, which likely is important based on the observation that PFAAs tend to preferentially distribute to liver and serum (Kemper, 2003; Kudo et al., 2007; Bogdanska et al., 2011; Chang et al., 2012; Sundstrom et al., 2012). The available experimental evidence supports the role of enterohepatic circulation as a possible contributing factor to the retention of PFHxS and PFOS in humans (Genuis et al., 2010; Genuis et al., 2013), and PFOS and PFOA in rats (Johnson et al., 1984). The latter studies investigated the effect of the binding agent, cholestyramine, in facilitating the gastrointestinal clearance of PFAAs.

In Chapter 3, we demonstrated that the uptake of PFBS, PFHxS and PFOS into freshly isolated human and rat hepatocytes was sodium-dependent and Na^+/taurocholate cotransporting polypeptide (NTCP), a sodium-dependent bile salt transporter expressed at the sinusoidal membrane of hepatocytes, was the responsible transporter (Zhao et al., 2015a). We have also shown that

bromosulfophthalein inhibits the sodium-independent portion of PFBS and PFHxS uptake into hepatocytes, indicating the involvement of sodium-independent transporters. At the hepatocyte sinusoidal membrane (Fig. 4-1), there are several organic anion transporting polypeptides (OATPs) expressed, including hOATP1B1, hOATP1B3 and hOATP2B1 in humans (Hsiang et al., 1999; König et al., 2000; Tamai et al., 2000; Roth et al., 2012) and rOATP1A1, rOATP1A4, rOATP1B2 and rOATP2B1 in rats (Bergwerk et al., 1996b; Noé et al., 1997; Kakyo et al., 1999; Nishio et al., 2000; Cattori et al., 2001). These are sodium-independent transporters that mediate the uptake of endogenous and exogenous compounds, such as taurocholate, estrone-3-sulfate, bilirubin and numerous drugs including statins (Hagenbuch and Stieger, 2013).

In addition to the liver, we also demonstrated that human apical sodium-dependent bile salt transporter (ASBT), but not rat ASBT, expressed at the apical membrane of the small intestine can transport PFOS, contributing to the intestinal reabsorption of PFOS in humans. However, transporters involved in the enterohepatic circulation of PFHxS and PFOS in rats and PFHxS in humans have not been identified. Candidate transporters in the human intestine are hOATP1A2 and hOATP2B1, but, based on recent proteomics work, only hOATP2B1 should be considered as an intestinal transporter (Fig.4-1) (Drozdzik et al., 2014). In rats, the corresponding transporters are rOATP1A5 and rOATP2B1 (Fig. 4-1). All three transporters are involved in the intestinal

absorption of several drugs including pravastatin, pitavastatin and fexofenadine (Kobayashi et al., 2003; Kikuchi et al., 2006; Shirasaka et al., 2010).

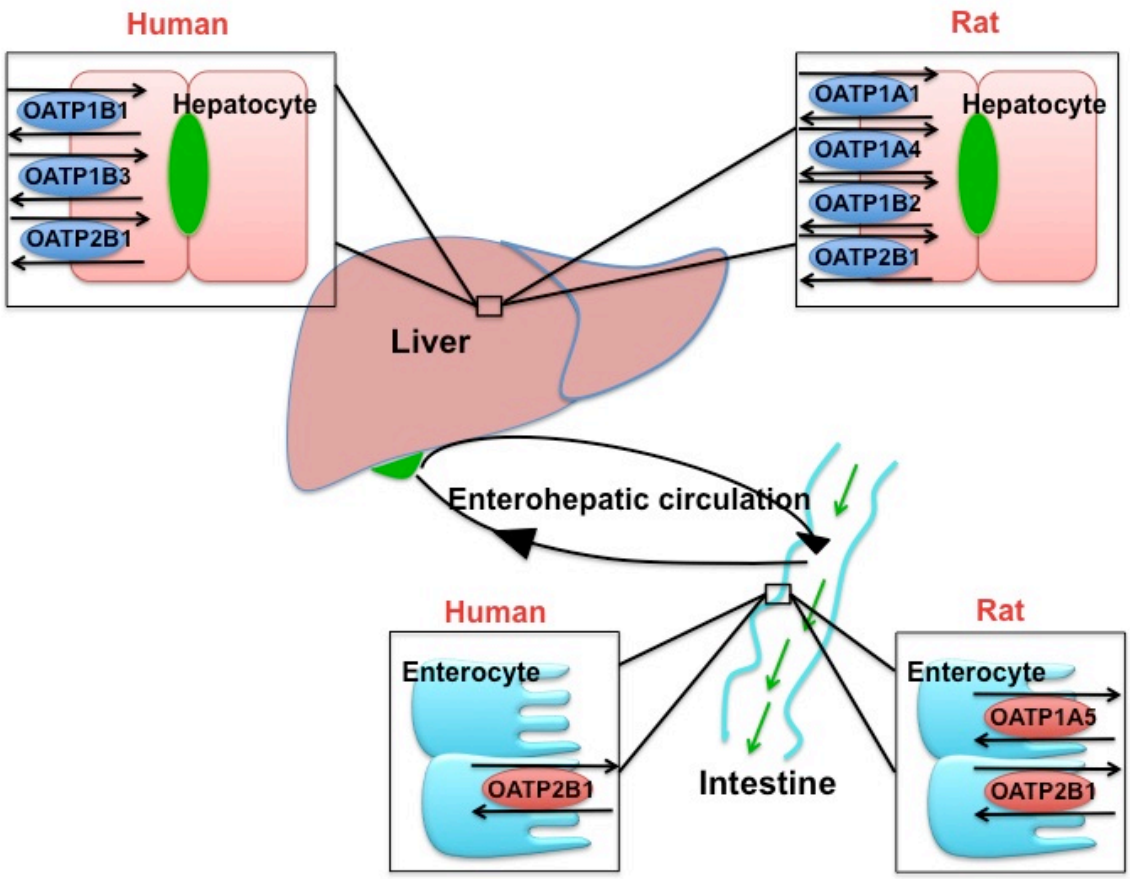

Figure 4-1: Expression of human and rat OATPs in hepatocytes and enterocytes

PFOA is a substrate of OATPs and OATs. In rats, rOATP1A1, rOAT1, rOAT3 and in humans, hOAT1, hOAT3, hOAT4 mediate the transport of PFOA (Katakura et al., 2007a; Nakagawa et al., 2008; Nakagawa et al., 2009; Yang et al., 2009). Consistently, our laboratory previously has shown chain-length dependent transport of PFCAs with 7 to 10 carbons by rOAT1, rOAT3 and rOATP1A1 (Weaver et al., 2010). Because rOATP1A1 is expressed at a higher level at the apical membrane of proximal tubules in male rats than in female rats (Lu et al., 1996) and seems to be involved in the reabsorption of PFOA, rOATP1A1-mediated reabsorption of PFOA is most likely the main mechanism for the longer half-life observed in male rats as opposed to females, as first speculated by Kudo et al (Kudo et al., 2002).

Based on these combined previous findings, we hypothesized that OATPs expressed in hepatocytes and in enterocytes can transport PFAAs and can contribute to the enterohepatic circulation and hepatic accumulation in humans and rats. Therefore, in the present study, we characterized the transport of PFBS, PFHxS, and PFOS and PFCAs with 7 to 10 carbons by the three human transporters hOATP1B1, hOATP1B3, hOATP2B1, and the transport of PFBS, PFHxS and PFOS by the rat transporters rOATP1A1, rOATP1A5, and rOATP1B2.

II. Results

2.1 Uptake of PFSAs by human OATP1B1 and OATP1B3

To determine whether PFSAs are substrates of the liver-specific transporters hOATP1B1 and hOATP1B3, uptake of 10 μM PFBS, PFHxS and PFOS was measured for 1 min using CHO cells stably expressing hOATP1B1 (Fig. 4-2A, B) or hOATP1B3 (Fig. 4-2C, D). The results demonstrate that PFBS, PFHxS and PFOS are substrates of both hOATP1B1 and hOATP1B3. Net uptake (transporter-mediated uptake) of PFHxS and PFOS was higher than of PFBS, while the signal-to-noise ratio for PFOS was lower than for the other two. Uptake was comparable between hOATP1B1 and hOATP1B3 except that for PFBS which was transported better by hOATP1B3.

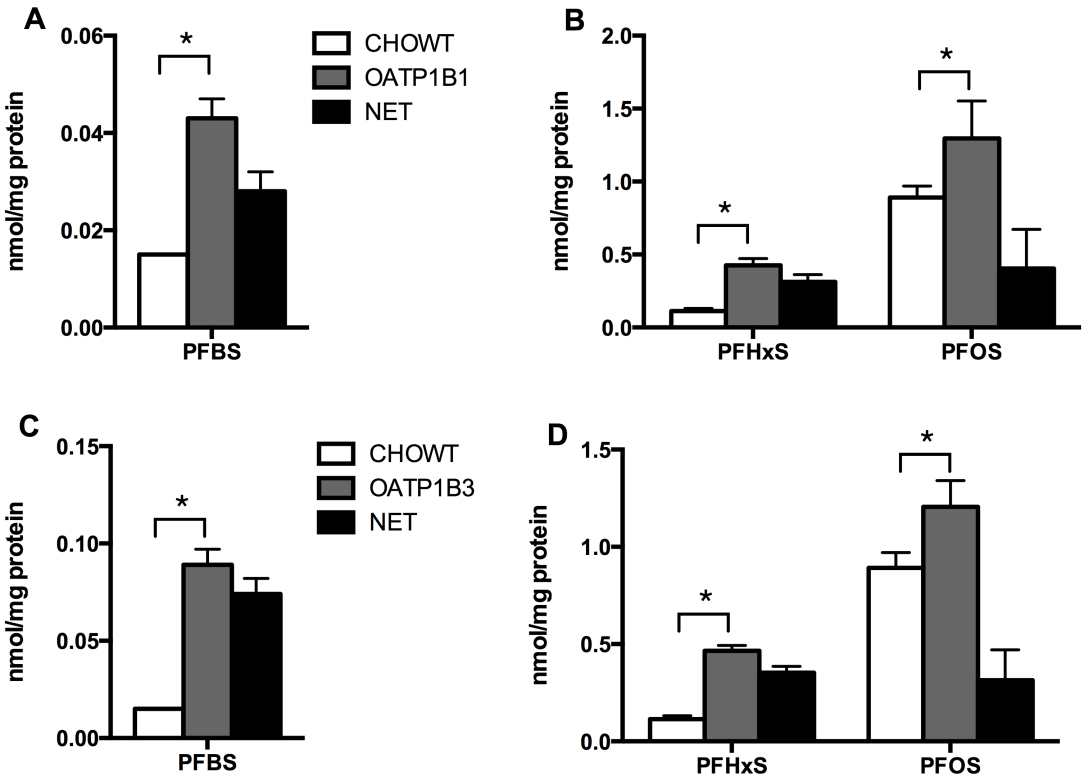

Figure 4-2: Uptake of PFSAs by hOATP1B1 (A, B) and hOATP1B3 (C, D)

CHO wild-type (white bars) or CHO cells stably expressing hOATP1B1 or hOATP1B3 (gray bars) were used to measure the uptake of 10 μM PFBS (A, C), PFHxS (B, D), and PFOS (B, D) for 1 min at 37°C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by wild-type cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD of triplicates from two independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well.

To further characterize the transport by hOATP1B1 and hOATP1B3, time-dependent uptake of PFBS, PFHxS and PFOS was measured at low (10 μM) and high (400 μM for PFBS and PFHxS, 200 μM for PFOS) substrate concentrations (Table 4-1). The initial linear range of hOATP1B1-mediated uptake of PFBS, PFHxS and PFOS was 2 min at low concentrations and 1 min at high concentrations. At low concentrations, hOATP1B3-mediated uptake of PFBS, PFHxS, and PFOS was linear up to 1, 5 and 2 min, respectively. At high concentrations, the initial linear range was 1, 2 and 2 min, respectively (Table 4-1). Based on these time-dependent uptake results, kinetics were performed at 40 sec for PFBS and PFHxS, and at 1.5 min for PFOS and the results are shown in Fig. 4-3A to 4-3F. The kinetic parameters were calculated based on the Michaelis-Menten equation and are summarized in Table 4-2. Each of the given PFSAs was transported with a similar affinity (K_m values) by the two OATPs. PFOS had the highest affinity (23 μM for hOATP1B1 and 32 μM for hOATP1B3) followed by PFBS (80 μM for hOATP1B1 and 63 μM for hOATP1B3) and PFHxS (101 μM for hOATP1B1 and 86 μM for hOATP1B3), respectively. In terms of capacity (V_{max} values) PFHxS was transported with the highest capacity (2.1 nmol/mg protein*min^{-1} for hOATP1B1 and 2.4 nmol/mg protein*min^{-1} for hOATP1B3), followed by PFOS (0.8 nmol/mg protein*min^{-1} for hOATP1B1 and 1.0 nmol/mg protein*min^{-1} for hOATP1B3) and PFBS (0.2 nmol/mg protein*min^{-1} for both hOATP1B1 and hOATP1B3), respectively. Dividing the V_{max} by the K_m value yields the transport efficiency (or transporter clearance) and suggested that PFHxS and PFOS are transported about tenfold more efficiently than PFBS

(Table 4-2), correlating with the longer serum elimination half-lives of PFHxS and PFOS as compared to PFBS.

Table 4-1: Summary of initial linear ranges of human and rat OATP-mediated uptake of PFBS, PFHxS, and PFOS

	Low concentration			High concentration		
Transporter	**Initial Linear Range (min)**			**Initial Linear Range (min)**		
	PFBS	PFHxS	PFOS	PFBS	PFHxS	PFOS
	10 µM	10 µM	10 µM	400 µM	400 µM	400 µM
hOATP1B1	2	2	2	1	1	1[1)]
hOATP1B3	1	5	2	1	2	2[1)]
hOATP2B1	2	1	5	1	1	1
rOATP1A1	nd[2)]	5	5	nd[2)]	5	5
rOATP1A5	1	10	10	2	1	5

[1)] PFOS concentration was 200 µM for this transporter; [2)] nd: not determined

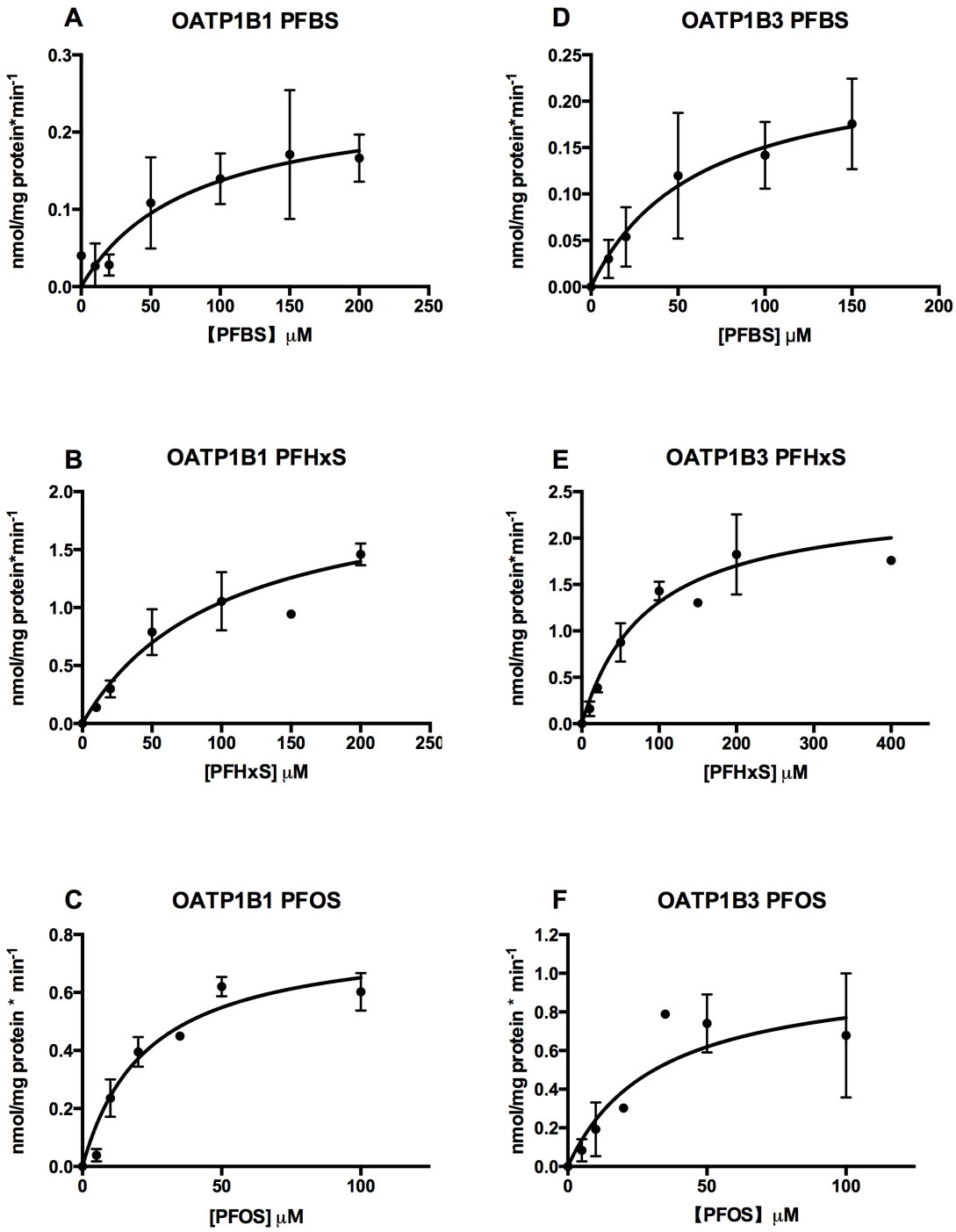
A
OATP1B1 PFBS
nmol/mg protein*min-1
0.3
0.2
0.1
0.0
0
50
100
150
200
250
【PFBS】μM
D
OATP1B3 PFBS
nmol/mg protein*min-1
0.25
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
[PFBS] μM
B
OATP1B1 PFHxS
nmol/mg protein*min-1
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
[PFHxS] μM
E
OATP1B3 PFHxS
nmol/mg protein*min-1
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
[PFHxS] μM
C
OATP1B1 PFOS
nmol/mg protein * min-1
0.8
0.6
0.4
0.2
0.0
0
50
100
[PFOS] μM
F
OATP1B3 PFOS
nmol/mg protein * min-1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
【PFOS】μM

Figure 4-3: Kinetics of hOATP1B1- (A, B, C) and hOATP1B3- (D, E, F) mediated transport of PFBS (A, D), PFHxS (B, E), and PFOS (C, F)

CHO wild-type, CHO-hOATP1B1 and hOATP1B3 cells were used to measure the uptake (at 40 seconds for PFBS and PFHxS, and 90 seconds for PFOS) of increasing concentrations of PFBS, PFHxS and PFOS. Net uptake was calculated by subtracting the values of uptake mediated by wild-type cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 4-2). The results were corrected for total protein concentration in each well. Each point represents the mean ± SD from three independent experiments performed in triplicate.

Table 4-2: Kinetic parameters of PFBS, PFHxS, and PFOS transport mediated by human OATP1B1, OATP1B3 and OATP2B1

Transporter	PFSA	K_m(μM)	V_{max} (nmol/mg protein*min^{-1})	V_{max}/K_m(μl/mg protein*min-^{1})
hOATP1B1	PFBS	79.9±46.6	0.2±0.06	2.5
	PFHxS	101±32.1	2.1±0.3	21
	PFOS	23.1±5.9	0.8±0.07	35
hOATP1B3	PFBS	62.5±36.7	0.2±0.07	3.2
	PFHxS	85.6±23.5	2.4±0.3	28
	PFOS	32.1±18.9	1.0±0.2	31
hOATP2B1	PFBS	122±57.1	0.6±0.1	4.9
	PFHxS	53.1±18.9	2.0±0.2	38
	PFOS	47.6±18.8	4.7±0.7	99

2.2 Uptake of PFSAs by human OATP2B1

In addition to hOATP1B1 and hOATP1B3, hOATP2B1 is also expressed at the basolateral membrane of hepatocytes and appears to be the major multispecific OATP expressed at the apical membrane of human enterocytes. To determine whether hOATP2B1 can transport PFSAs, uptake of 10 μM PFBS, PFHxS and PFOS was measured at 2 min using CHO Flp-in cells stably expressing hOATP2B1 (Fig. 4-4A, B). Net transporter-mediated uptake of PFOS was the highest, followed by PFHxS and PFBS.

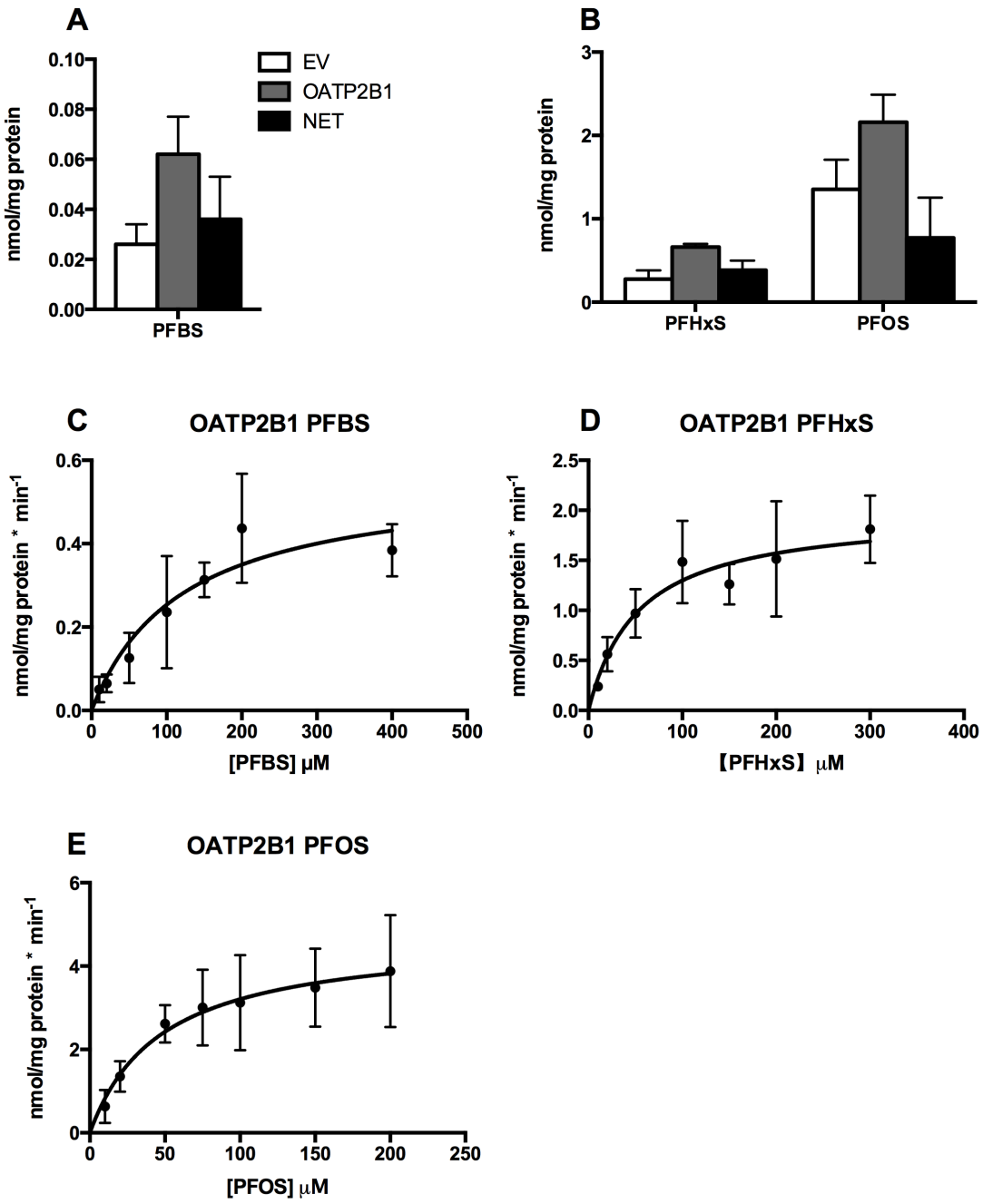
A
EV
OATP2B1
NET
nmol/mg protein
0.10
0.08
0.06
0.04
0.02
0.00
PFBS
B
nmol/mg protein
3
2
1
0
PFHxS
PFOS
C
OATP2B1 PFBS
nmol/mg protein * min-1
0.6
0.4
0.2
0.0
0
100
200
300
400
500
[PFBS] μM
D
OATP2B1 PFHxS
nmol/mg protein * min-1
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
【PFHxS】μM
E
OATP2B1 PFOS
nmol/mg protein * min-1
6
4
2
0
0
50
100
150
200
250
[PFOS] μM

Figure 4-4: Uptake of PFSAs by hOATP2B1

CHO Flp-in cells transfected with pcDNA5/FRT (EV) or stably expressing hOATP2B1 were used to measure the uptake of PFBS (A, C), PFHxS (B, D), and PFOS (B, F). A, B） Uptake of 10 μM PFBS, PFHxS and PFOS was determined at 37 °C for 2 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by CHO Flp-In EV cells from uptake mediated by transporter-expressing cells. C, D, E) Uptake of increasing concentrations of PFBS, PFHxS and PFOS was determined at 37 °C for 1 min. Net uptake was calculated by subtracting the values of uptake mediated by Flp-In EV cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 4-2). Each point represents the mean ± SD from three independent experiments performed in triplicate. The results were corrected for total protein concentration in each well.

Next, time-dependent uptake of PFBS, PFHxS and PFOS was measured at low (10 μM) and high (400 μM) substrate concentrations to determine the initial linear range (Table 4-1). At low substrate concentrations, hOATP2B1-mediated uptake of PFBS, PFHxS and PFOS was linear up to 2, 1 and 5 min, respectively. At high substrate concentrations, uptake for all three substrates was linear only for 1 min. Therefore, concentration-dependent uptake of all three PFSAs was measured at 1 min, and the results are summarized in Fig.4-4C to 4-4E. After correcting for empty-vector control cells the data were fitted using non-linear regression analysis to the Michaelis-Menten equation, and the calculated kinetic parameters (K_m and V_{max}) are shown in (Table 4-2). PFOS and PFHxS were transported by hOATP2B1 with higher affinity (K_m = 48 and 53 μM, respectively) than PFBS (122 μM). However, bigger differences were seen for the respective V_{max} values. Transport of PFOS had the largest capacity (4.7 nmol/mg protein*min^{-1}) followed by PFHxS (2.0 nmol/mg protein*min^{-1}) and PFBS (0.6 nmol/mg protein*min^{-1}). As a consequence, transport efficiency for PFOS was twice as high as for PFHxS and even twenty times higher than for PFBS (Table 4-2). Again, these numbers correlated well with the longer serum elimination half-lives for PFHxS and PFOS as compared to PFBS.

Previous studies demonstrated that transport by hOATP2B1 is pH-dependent (Nozawa et al., 2004). However, no significant difference was observed for the transport of the three PFSAs by hOATP2B1 when uptake was measured at pH 7.4 or pH 5.5 (data not shown).

2.3 Uptake of PFSAs by rat liver OATPs

In rats, rOATP1A1, rOATP1A4, rOATP1B2, and rOATP2B1 are all expressed at the basolateral membrane of hepatocytes. To determine which of these OATPs are involved in the liver accumulation of PFSAs in rats, uptake of 10 μM PFBS, PFHxS, and PFOS was measured in HEK293 cells transiently expressing these transporters individually. As shown in Figure 4-5A and 4-5B, rOATP1A1 could transport all three PFSAs. Because the signals were well above the empty vector transfected cells for PFHxS and PFOS, kinetics were performed at a 5 min uptake point (within the initial linear range, Table 4-1) and the results are summarized in Figure 4-5C, 4-5D and in Table 4-3. The capacity for PFHxS and PFOS transport was similar (1.0 nmol/mg protein*min^{-1} for PFHxS and 0.7 nmol/mg protein*min^{-1} for PFOS), but the affinity for PFOS (K_m = 37 μM) was about 7 fold higher than for PFHxS (K_m = 256 μM) (Table 4-3).

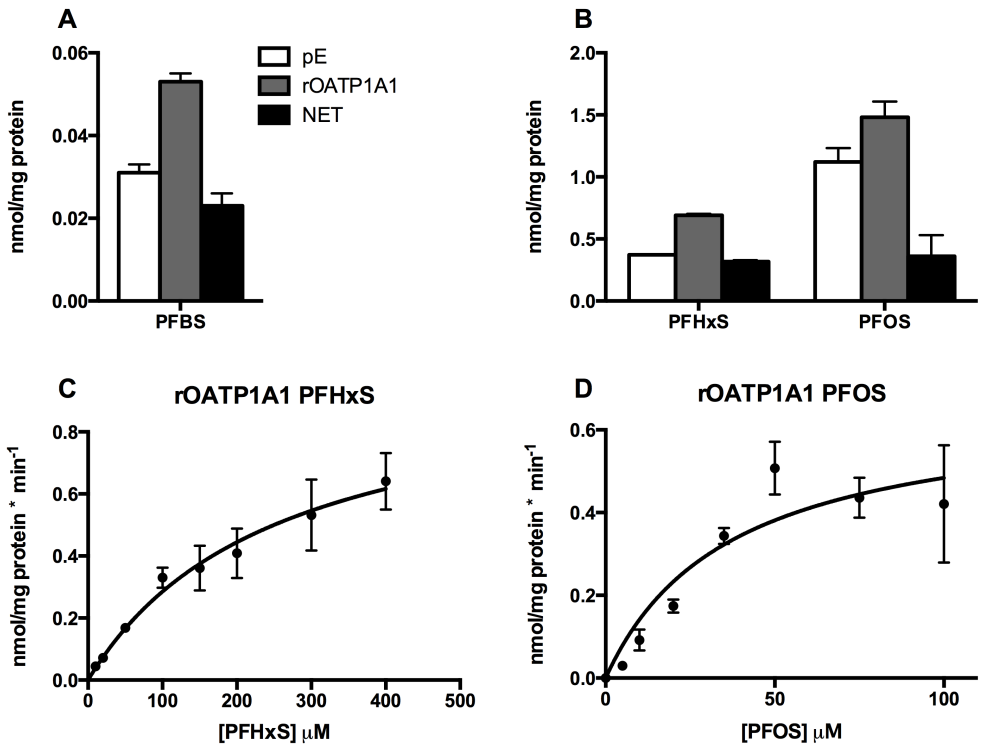

Figure 4-5: Uptake of PFSAs by rOATP1A1

HEK293 cells transiently transfected with empty vector pExpress-1 (pE) or rOATP1A1 were used to measure the uptake of PFBS (A), PFHxS (B, C), and PFOS (B, D). A, B） Uptake of 10 μM PFBS, PFHxS and PFOS was measured at 37 °C for 1 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by pE transfected HEK293 cells from uptake mediated by transporter-expressing cells. C, D) Uptake of increasing concentrations of PFHxS and PFOS was determined at 37 °C for 5 min. Net uptake was calculated by subtracting the values of uptake mediated by cells transfected with pE from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 4-3). Each point represents the mean ± SD from three independent experiments performed in triplicate. The results were corrected for total protein concentration in each well.

Table 4-3: Kinetic parameters of PFBS, PFHxS and PFOS transport mediated by rat OATP1A1 and OATP1A5

Transporter	PFSA	K_m (μM)	V_{max} (nmol/mg protein*min^{-1})	V_{max}/K_m (μl/mg protein*min-^{1})
rOATP1A1	PFHxS	256±68.9	1.0±0.1	3.9
	PFOS	36.9±18.7	0.7±0.1	19
rOATP1A5	PFBS	117±41.0	0.2±0.04	1.7
	PFHxS	160±122	1.2±0.5	7.5
	PFOS	55.0±18.4	1.2±0.1	22

Results for the uptake of 10 μM PFBS, PFHxS and PFOS at 1 min and 5 min by rOATP1B2 and rOATP2B1 are shown in Fig. 4-6. All three PFAAs are transported by both transporters, and the signals were comparable between rOATP1B2 and rOATP2B1. For both OATPs, the net transporter-mediated uptake of PFBS was about tenfold lower than uptake of PFHxS and PFOS. However, signal to background ratios were not high enough to perform kinetic analyses. For the fourth rat OATP, rOATP1A4, no net transporter-mediated uptake could be detected at either 1 min or 5 min for any of the PFAAs (data not shown).

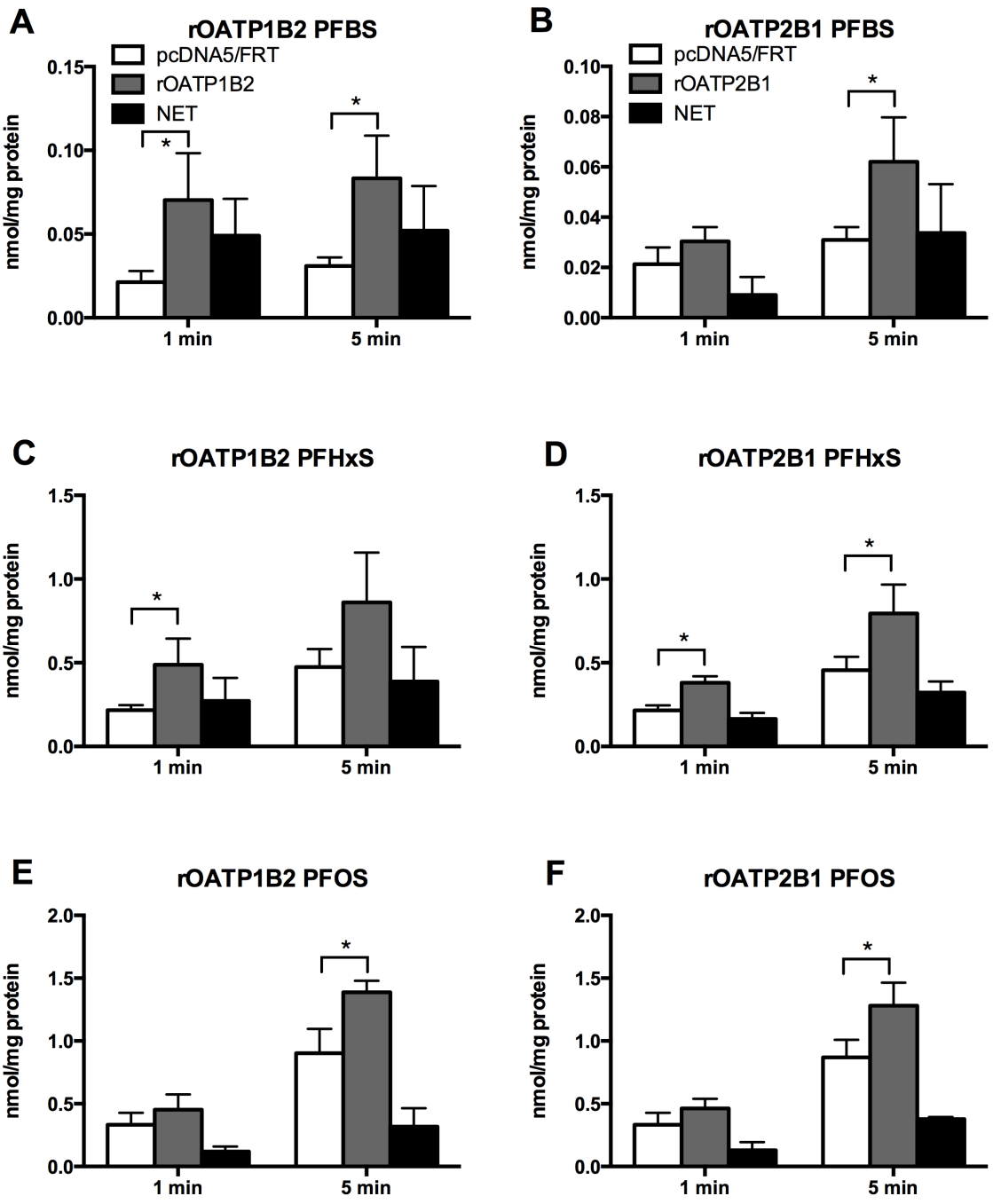
A
rOATP1B2 PFBS
pcDNA5/FRT
rOATP1B2
NET
nmol/mg protein
0.15
0.10
0.05
0.00
1 min
5 min
*
B
rOATP2B1 PFBS
pcDNA5/FRT
rOATP2B1
NET
nmol/mg protein
0.10
0.08
0.06
0.04
0.02
0.00
1 min
5 min
*
C
rOATP1B2 PFHxS
nmol/mg protein
1.5
1.0
0.5
0.0
1 min
5 min
*
D
rOATP2B1 PFHxS
nmol/mg protein
1.5
1.0
0.5
0.0
1 min
5 min
*
E
rOATP1B2 PFOS
nmol/mg protein
2.0
1.5
1.0
0.5
0.0
1 min
5 min
*
F
rOATP2B1 PFOS
nmol/mg protein
2.0
1.5
1.0
0.5
0.0
1 min
5 min
*

Figure 4-6: Uptake of PFSAs by rOATP1B2 (A, C, E) and rOATP2B1 (B, D, F)

HEK293 cells transiently transfected with empty vector pcDNA5/FRT (white bars), and rOATP1B2 or rOATP2B1 (grey bars) were used to measure the uptake of 10 μM PFBS, PFHxS or PFOS for 1 min and 5 min at 37°C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by empty vector transfected cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD from three independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. * $p < 0.05$.

2.4 Uptake of PFSAs by rat OATP1A5

Besides rOATP2B1, rOATP1A5 is also expressed on the apical membrane of enterocytes (Walters et al., 2000a). To test whether rOATP1A5 could also contribute to the disposition (absorption) of PFSAs, HEK293 cells transiently expressing rOATP1A5 were used to measure the uptake of 10 µM PFBS, PFHxS or PFOS. As summarized in Figures 4-7A and 4-7B, rOATP1A5 transports all three PFSAs. Net rOATP1A5-mediated uptake was highest for PFOS, followed by PFHxS and PFBS.

In order to perform kinetics analyses, first, time-dependent uptake was measured at low (10 µM) and high (400 µM) substrate concentrations (Table 4-1). Based on these experiments, the concentration-dependent uptake of PFBS and PFHxS was measured at 1 min, while for PFOS, 5 min were used. As shown in Table 4, PFOS was transported with the highest affinity (K_m = 55 µM), followed by PFBS (K_m = 117 µM) and by PFHxS (K_m = 160 µM). In terms of capacity, PFHxS and PFOS were transported with the same V_{max} value (1.2 nmol/mg protein*min^{-1}), while PFBS was transported with a sixfold lower capacity. As a consequence, transporter efficiency for PFOS was threefold higher than for PFHxS and thirteen-fold higher than for PFBS.

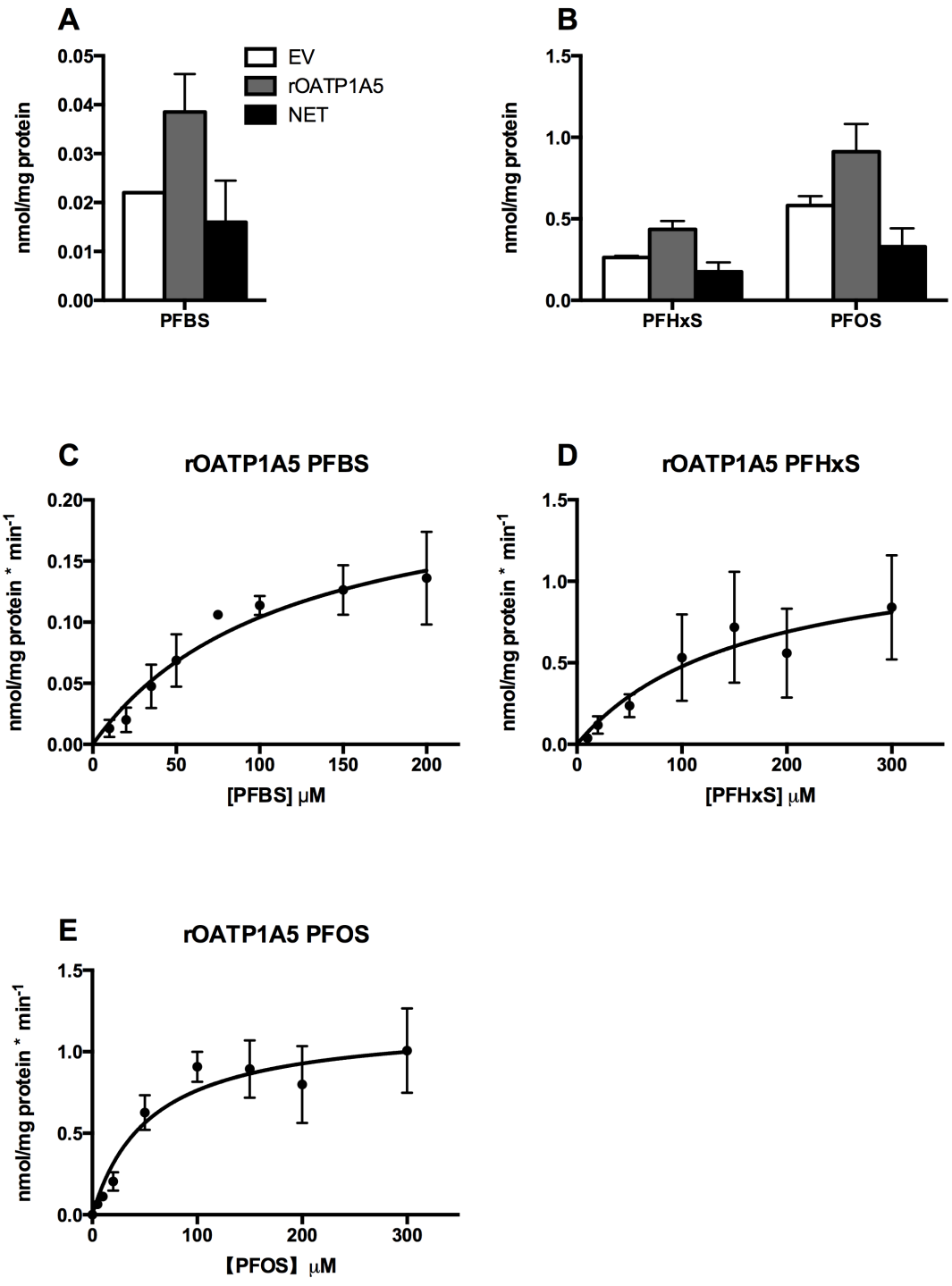
A
EV
rOATP1A5
NET
nmol/mg protein
0.05
0.04
0.03
0.02
0.01
0.00
PFBS
B
nmol/mg protein
1.5
1.0
0.5
0.0
PFHxS
PFOS
C
rOATP1A5 PFBS
nmol/mg protein * min-1
0.20
0.15
0.10
0.05
0.00
0
50
100
150
200
[PFBS] μM
D
rOATP1A5 PFHxS
nmol/mg protein * min-1
1.5
1.0
0.5
0.0
0
100
200
300
[PFHxS] μM
E
rOATP1A5 PFOS
nmol/mg protein * min-1
1.5
1.0
0.5
0.0
0
100
200
300
【PFOS】 μM

Figure 4-7: Uptake of PFSAs by rOATP1A5

HEK293 cells transiently transfected with empty pSport6 vector (EV) or rOATP1A5 were used to measure the uptake of PFBS (A, C), PFHxS (B, D), and PFOS (B, E). A,B） Uptake of 10 μM PFBS, PFHxS and PFOS was measured at 37 °C for 1 min. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by EV transfected HEK293 cells from uptake mediated by transporter-expressing cells. C, D, E) Uptake of increasing concentrations of PFBS, PFHxS and PFOS was measured at 37 °C for 1 (PFBS and PFHxS) or 5 min (PFOS). Net uptake was calculated by subtracting the values of uptake mediated by EV transfected cells from uptake mediated by transporter-expressing cells. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 4-3). Each point represents the mean ± SD from three independent experiments performed in triplicates. The results were corrected for total protein concentration in each well.

2.5 Uptake of perfluoroalkyl carboxylates by human OATP1B1, OATP1B3 and OATP2B1

To test which OATPs can contribute to the transport of perfluoroheptanoate (C7), perfluorooctanoate (C8), perfluorononanoate (C9), and perfluorodecanoate (C10) into human hepatocytes, 1 min uptake of 10 µM of C7 to C10 was measured in CHO cells stably expressing hOATP1B1, hOATP1B3 or hOATP2B1. The results are summarized in Fig. 4-8 and demonstrate that C8 is a substrate of all three human OATPs that are expressed in hepatocytes. C10 is transported by hOATP1B1 and hOATP1B3 while C7 and C9 are only transported by hOATP1B1. Transport mediated by hOATP1B3 was highest and similar for C8 and C10. In contrast, hOATP1B1-mediated transport increased with the chain-length from C7 to C9, while uptake of C10 was lower. Uptake of C8 mediated by hOATP2B1 was similar to the uptake mediated by hOATP1B1.

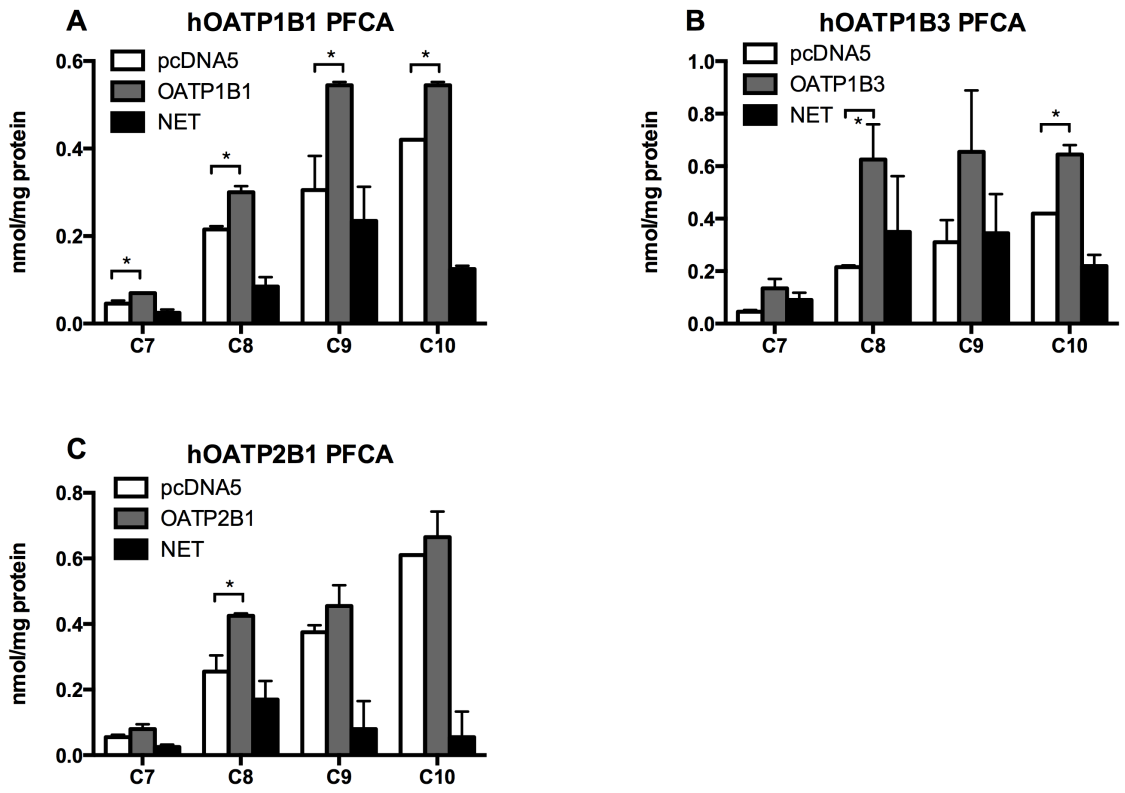

Figure 4-8: Uptake of PFCAs by hOATP1B1 (A), hOATP1B3 (B) and hOATP2B1 (C)

CHO wild-type cells (white bars) or CHO cells stably expressing hOATP1B1, hOATP1B3 or hOATP2B1 (gray bars) were used to measure the uptake of 10 µM C7-C10 PFCA for 1 min at 37°C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by wild-type cells from uptake mediated by transporter-expressing cells. Each bar represents the mean ± SD from two independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. * $p < 0.05$.

III. Discussion

In this chapter, we have demonstrated that PFAAs, including PFBS, PFHxS and PFOS are substrates of human hOATP1B1, hOATP1B3, hOATP2B1 and rat rOATP1A1, rOATP1B2, rOATP2B1 and rOATP1A5. Furthermore, we showed that hOATP1B1, hOATP1B3 and hOATP2B1 can transport certain PFCAs with 7 to 10 carbons.

In the studies presented in Chapter 3, we demonstrated that bromosulfophthalein inhibits sodium-independent uptake of PFBS and PFHxS into human and rat hepatocytes, while sodium-independent uptake of PFOS was not inhibited (Zhao et al., 2015a). Those results suggested that, besides the sodium-dependent portion mediated by hNTCP or rNTCP, a sodium-independent portion of uptake at least for PFHxS and PFBS exists. Our findings reported in this study clearly demonstrate that the human hOATP1B1, hOATP1B3, and hOATP2B1, as well as the rat rOATP1A1, rOATP1B2, and rOATP2B1, can transport PFBS and PFHxS and therefore likely contribute to the sodium-independent uptake into human and rat hepatocytes. Although there was no inhibition of sodium-independent PFOS uptake by bromosulfophthalein, direct uptake measurements showed that all three human OATPs were able to transport PFOS. Comparing the calculated transport efficiencies (V_{max}/K_m values) for the individual OATPs with respect to the three different PFSAs, our data clearly indicated that PFBS was transported with the lowest efficiency by all three human OATPs (Table 4-1). Transport efficiencies for PFHxS and PFOS were almost ten fold higher than PFBS for all

three human OATPs, correlating with the much longer serum elimination half-lives of PFHxS and PFOS. Thus in addition to hNTCP, hOATP1B1, hOATP1B3 and hOATP2B1 can contribute to the enterohepatic circulation of PFHxS and PFOS and seem to contribute much less to the enterohepatic circulation of PFBS.

Human OATP2B1 is expressed in hepatocytes (Prasad et al., 2014; Peng et al., 2015), and also at the apical membrane of human enterocytes (Drozdzik et al., 2014). It can mediate the uptake of steroid hormones, drugs, and other xenobiotics (Roth et al., 2012) and hence facilitate their enterohepatic circulation. Our findings that hOATP2B1 can transport all three PFSAs suggest that, in addition to the previously reported hASBT (Zhao et al., 2015a), hOATP2B1 also mediates the uptake of PFOS into enterocytes and thus contributes to the enterohepatic circulation of PFOS. Furthermore, since hASBT cannot transport PFHxS (Zhao et al., 2015a), hOATP2B1 is the first human transporter expressed in the intestine that is reported to mediate the reabsorption of PFHxS.

In addition to these human liver OATPs, we also identified three OATPs expressed in rat livers, rOATP1A1, rOATP1B2, and rOATP2B1, that are able to mediate the uptake of PFBS, PFHxS and PFOS. Rat OATP1A1 transported PFHxS with a similar affinity (K_m = 256 μM) as rNTCP (K_m = 294 μM). Its affinity to PFOS (K_m = 37 μM) was much higher and close to the affinities of the human liver OATPs. Rat OATP1A1 is also expressed in the apical membrane of renal proximal tubular epithelial cells (Bergwerk et al., 1996b), where it can mediate

the reabsorption of organic anions in exchange for intracellular reduced glutathione (Li et al., 1998). In addition, rOATP1A1 is expressed in a male-dominant manner (Kato et al., 2002). We previously demonstrated that rOATP1A1 transports several PFCAs, such as PFOA, and hence, at least partly, contributes to the longer serum elimination half-life of PFOA in male rats (Weaver et al., 2010). As for PFHxS, pharmacokinetic studies revealed that the elimination half-life is much slower in male rats ($T_{1/2}$ = 29.1 days) than in female rats ($T_{1/2}$ = 1.6 days) (Sundstrom et al., 2012). Increased reabsorption of PFHxS in male rats via rOATP1A1 could be part of the mechanism for this gender-specific difference in elimination. Although rOATP1A1 can also transport PFOS, the elimination half-life of PFOS in male rats is somewhat shorter than in female rats (Chang et al., 2012), indicating that rOATP1A1 is not the major transporter for the renal elimination of PFOS.

Enterohepatic circulation of PFOS was not only observed in humans, but also in rats (Johnson et al., 1984). So far the mechanism for intestinal reabsorption of PFOS, and also of PFBS and PFHxS, in rats could not be explained because, unlike in humans, rASBT does not transport these PFSAs (Zhao et al., 2015a). In the present study we report, for the first time, that rOATP1A5 and rOATP2B1, OATPs expressed in rat intestine, transport all three PFSAs. Thus, reabsorption of PFOS via rOATP1A5 and rOATP2B1 offers an explanation as to how PFOS can be kept in the enterohepatic circulation in the absence of rASBT-mediated transport.

Differential renal clearance was observed for PFCA and PFSA analogs with different chain-lengths according to pharmacokinetic studies in animal models (Olsen et al., 2009; Chang et al., 2012; Han et al., 2012; Sundstrom et al., 2012). The transport of PFSAs was also chain-length dependent. As we discussed previously, NTCP transported PFSAs with decreasing affinity but increasing capacity as the chain-length increased. The opposite trend was seen for OATP-mediated uptake. For all five of the OATPs where kinetic analysis was possible, PFOS was transported with the highest affinity, followed by PFBS and PFHxS which were transported with similar but clearly lower affinities. Nevertheless, transport efficiency generally increased with the increase in chain-length because V_{max} values were always higher for PFHxS and PFOS. As indicated before, this higher transport efficiency could well be, at least in part, the distinguishing mechanism for the much shorter half-life of PFBS compared to the longer half-lives of PFHxS and PFOS.

We have previously shown that rOATP1A1 can transport the perfluoroalkyl carboxylates C7 to C10 (Weaver et al., 2010) and contributes to the gender-specific renal elimination of C8 to C10. Given that rOATP1A1 is also expressed in the liver, we tested in the current study whether any of the OATPs expressed in human hepatocytes would transport these carboxylates and thus could contribute to the long half-lives of these chemicals (Olsen et al., 2007) by helping to keep them within the enterohepatic circulation in humans. The fact that all three OATPs expressed in human hepatocytes can transport all or some of the

four PFCAs, and that the two major liver OATPs, hOATP1B1 and hOATP1B3, transport the longer chain C8 to C10 better than the shorter C7 support a potential role of these OATPs in the longer serum elimination half-lives of these PFCAs.

In conclusion, our studies demonstrate that PFBS, PFHxS and PFOS are substrates of several OATPs (hOATP1B1, hOATP1B3, hOATP2B1, rOATP1A1, rOATP1B2, rOATP2B1 and rOATP1A5) expressed in hepatocytes and enterocytes in humans and rats. Furthermore, PFCAs with 7 to 10 carbons are substrates of hOATP1B1. In addition, hOATP1B3 transports at least C8 and C10, and hOATP2B1 transports C8. Thus, the sodium-independent transporters of the OATP family serve as additional mediators to facilitate the enterohepatic circulation of PFHxS and PFOS in humans and rats and rOATP1A1 might contribute to the gender-specific renal elimination of PFHxS in rats.

CHAPTER 5

OAT1 and OAT3 are involved in the renal clearance of PFSAs in rats

I. Introduction

Xenobiotics with molecular weight higher than 450 Da tend to enter the liver and undergo biliary excretion. In contrast, xenobiotics with molecular weight lower than 350 Da are more likely to be eliminated through urine (Hirom, 1976). Pharmacokinetic studies in laboratory animals revealed that PFASs with long chain-length (PFOS; C8; 500 Da) are excreted through feces, with short chain-length (PFBS; C4; 300 Da) through urine and with intermediate chain-length (PFHxS; C6; 400 Da) through both routes (Olsen et al., 2009; Chang et al., 2012; Sundstrom et al., 2012).

Glomerular filtration and tubular secretion in the kidney are the major mechanisms involved in the excretion of water-soluble undesired endogenous metabolites and exogenous compounds, while tubular reabsorption prevents the loss of useful solutes and metabolites. With respect to tubular secretion and reabsorption of organic anions, transporters expressed in the proximal tubules play an important role. Transporter families involved in the transport of organic anions in the kidney include the organic anion transporter (OAT) family, the organic anion transporting polypeptide (OATP) superfamily, and the multidrug resistance associated protein (MRP) superfamilies (Sekine et al., 2006; You and

Morris, 2007). Members of all these families are multispecific transporters that mediate the transport of a broad range of structurally diverse compounds and have overlapping substrate specificities within the families (Roth et al., 2012). Proximal tubular epithelial cells are polarized epithelial cells with the basolateral membrane facing the blood and the apical membrane facing the tubular lumen. There are specific transporters expressed on both sides as shown in Fig 5-1.

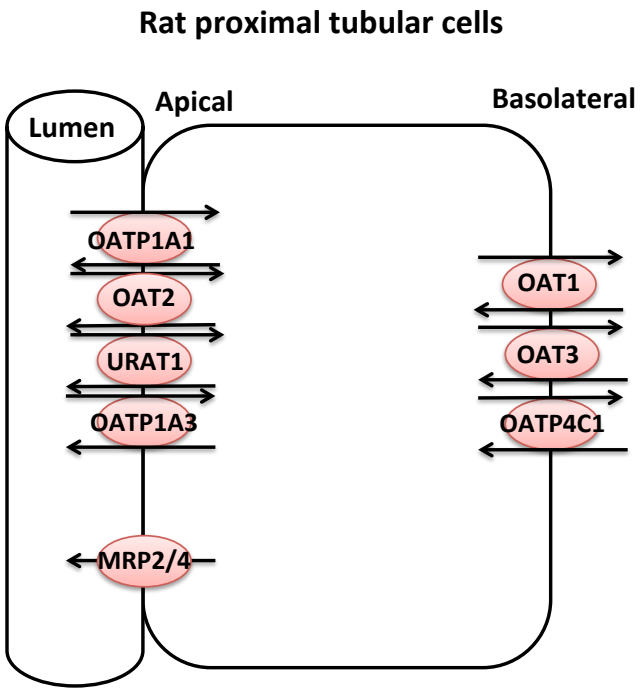

Figure 5-1: Transporters for organic anions expressed in proximal tubular epithelial cells of rat kidney

At the apical membrane of proximal tubules of rat kidney, OATP1A1 (Bergwerk et al., 1996a), OATP1A3 (Masuda et al., 1997; Masuda et al., 1999), OAT2 (Kojima et al., 2002), OAT5 (Anzai et al., 2005), and URAT1 (Rizwan and Burckhardt, 2007) are uptake transporters mediating the reabsorption of organic anions from the urine while MRP2 (Schaub et al., 1999) and MRP4 (van Aubel et al., 2002) are responsible for secretion. Additional uptake transporters including OAT1, OAT3 (Tojo et al., 1999; Kojima et al., 2002) and OATP4C1 (Kuo et al., 2012) expressed at the basolateral membrane are also involved in the secretion of organic anions.

Transporters expressed in the kidney and regulated by sex hormones play a role in the gender-dependent clearance of PFASs (Kudo et al., 2002). Several human and rat transporters mediate the uptake of PFCAs *in vitro*. PFOA is a substrate of renal uptake transporters, including human and rat OAT1, OAT3, URAT1, human OAT4, as well as rat OAT2 and OATP1A1 (Katakura et al., 2007b; Nakagawa et al., 2008; Weaver et al., 2010; Yang et al., 2010). In addition to PFOA, our group showed that rat OAT1, OAT3 and OATP1A1 could also transport PFCA with 7, 9 and 10 carbons (Weaver et al., 2010). Although not absolutely conclusive, OATP1A1 seems to be the major contributor to the gender-dependent renal elimination of PFCAs in rats because its higher expression levels in males would allow OATP1A1, which is expressed at the apical membrane to reabsorb more filtered and secreted PFCAs than in females. We and others also reported that inhibition of the model substrate uptake by these transporters is chain length-dependent, suggesting a role of transporters in the chain length-dependent clearance (Weaver et al., 2010; Yang et al., 2010). Given that the expression of specific transporters differs among species, it is reasonable to speculate that transporter studies will explain the species differences in the renal clearance of PFSAs.

To determine whether any of the OATs expressed in the proximal tubular basolateral membrane are involved in the renal secretion of PFSAs in rats, we measured the transport of PFBS, PFHxS and PFOS by rOAT1 and rOAT3 transiently expressed in HEK293 cells. We found that PFBS and PFHxS are

substrates of both rOAT1 and rOAT3. Based on these results, further kinetic characterizations were performed.

II. Results

2.1 Uptake of PFSAs by rat OAT1 and OAT3

To determine whether PFSAs are substrates of the rat kidney transporters rOAT1 and rOAT3, uptake of 10 μM PFBS, PFHxS and PFOS was measured at 1 min using HEK293 cells transiently expressing rOAT1 or rOAT3. As shown in Fig 5-2, rat OAT1 and OAT3 transport PFBS and PFHxS, but not PFOS. Net uptake of PFHxS was higher than uptake of PFBS while the signal-to-noise ratio was greater for PFBS. Rat OAT3 mediated uptake had a slightly higher signal than uptake mediated by rOAT1.

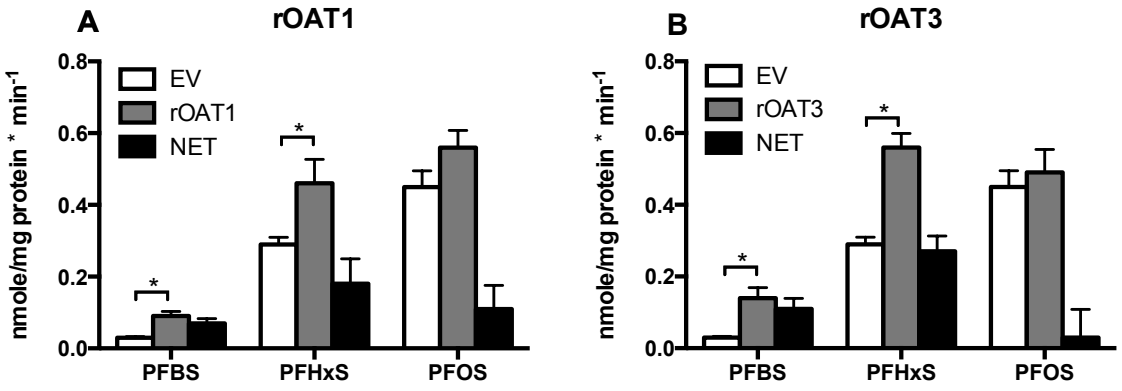

Figure 5-2: Uptake of PFSAs by rOAT1 (A) and rOAT3 (B)

HEK293 cells transiently transfected with empty vector pExpress-1(EV, white bars) and rOAT1 or rOAT3 (grey bars) were used to measure the uptake of 10 μM PFBS, PFHxS, and PFOS for 1 min at 37°C. Net uptake (black bars) was calculated by subtracting the values of uptake mediated by EV transfected cells from uptake mediated by transporter transfected cells. Each bar represents the mean ± SD from two independent experiments each performed with triplicate determinations. The results were corrected for total protein concentration in each well. *p<.05.

2.2 Kinetic determinations of PFBS and PFHxS by rat OAT1 and OAT3

To further characterize the transport by rOAT1 and rOAT3, time-dependent uptake of PFBS and PFHxS at low (10 μM) and high (400 μM) substrate concentration was measured. At low substrate concentration, the uptake by rOAT1 was linear up to 1 min for PFBS and to 30 sec for PFHxS (Fig 5-3A and 5-3B). Rat OAT3 mediated uptake of PFBS and PFHxS was linear up to 1 min and 2 min, respectively (Fig 5-3C and 5-3D). At high substrate concentrations, uptake for both substrates by both transporters was linear up to 30 sec (data not shown).

To stay within the initial linear range, concentration-dependent uptake of PFBS and PFHxS by rOAT1 and rOAT3 was measured at 20 sec. As can be seen in Fig 5-4, uptake was saturable. The kinetic parameters were calculated based on the Michaelis-Menten equation and are summarized in Table 5-1. The transport of PFBS mediated by rOAT1 has the lowest affinity (K_m=285 μM) and slowest intrinsic clearance (V_{max}/K_m=9.8 μl/mg protein*min^{-1}). Transport of PFHxS by rOAT1 (K_m=72.7 μM, V_{max}=4.1 nmol/mg protein*min^{-1}) has highest capacity with similar affinity as transport of PFBS (K_m=57.0 μM) and PFHxS (K_m=48.5 μM) by rOAT3. Both rOAT1 and rOAT3 transport PFHxS with greater affinity and higher capacity than PFBS.

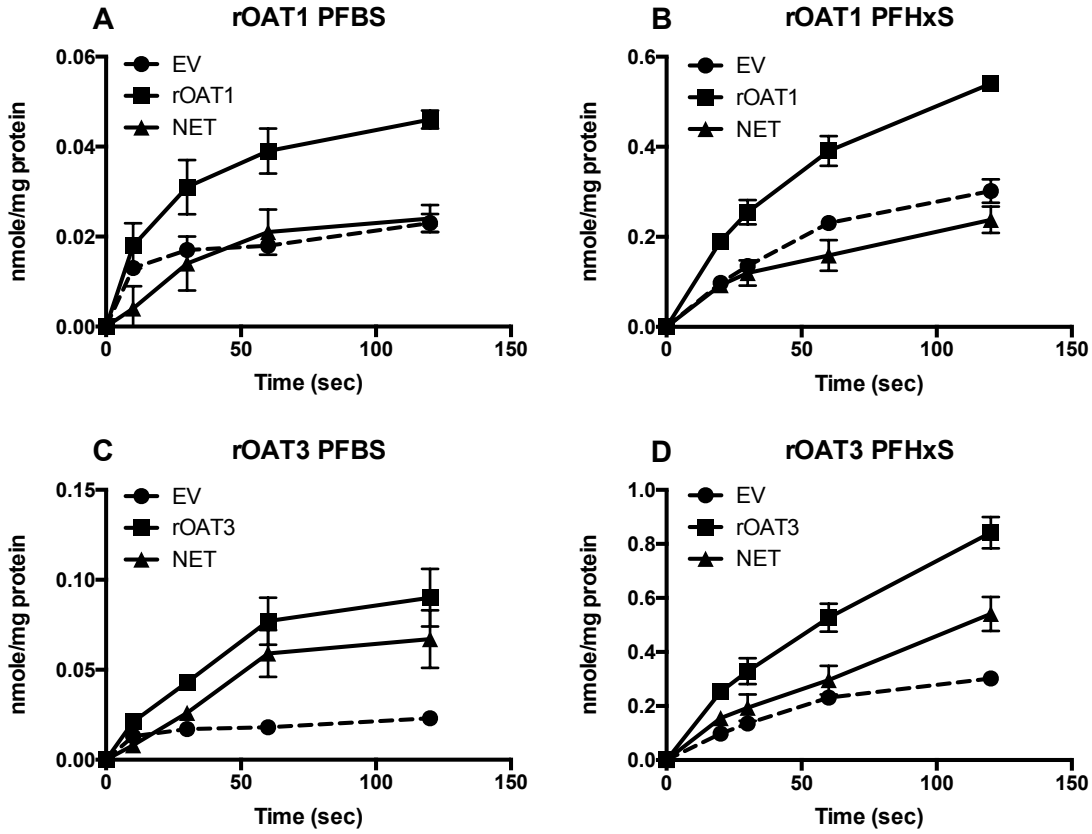

Figure 5-3: Time-dependent uptake of PFSAs by rOAT1 (A, B) and rOAT3 (C, D)

Uptake of 10 μM PFBS (A, C) and PFHxS (B, D) was measured at 37°C at the indicated time points using HEK293 cells transiently transfected with EV (circles), rOAT1 or rOAT3 (squares). Net uptake (triangles) was calculated by subtracting the value of uptake mediated by EV transfected cells from uptake mediated by transporter transfected cells. The results were corrected for total protein concentration in each well. Each point represents the mean ±SD of triplicates.

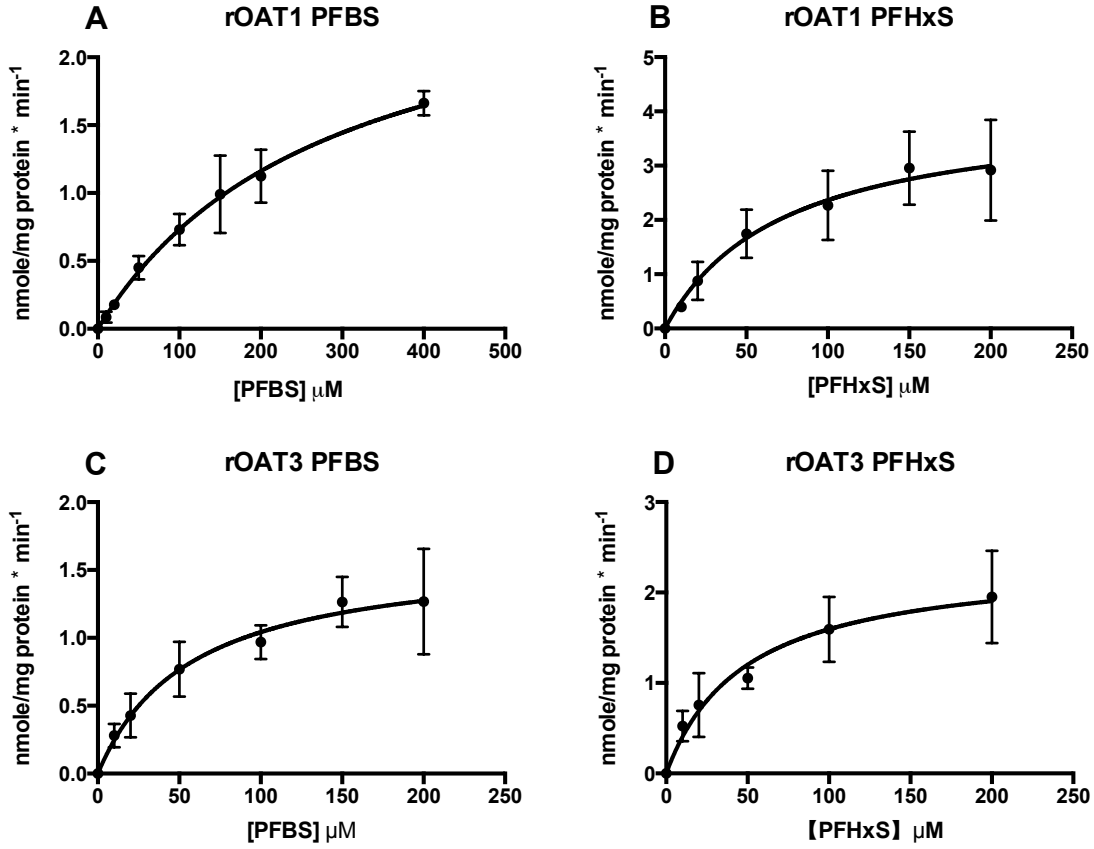

Figure 5-4: Kinetics of rOAT1- (A, B) and rOAT3- (C, D) mediated transport of PFBS (A, C) and PFHxS (B, D)

HEK293 cells transiently transfected with EV, rOAT1 or rOAT3 were used to measure the uptake of increasing concentrations of PFBS and PFHxS. Net uptake was calculated by subtracting the values of uptake mediated by EV transfected cells from uptake mediated by transporter transfected cells. Resulting data were fitted to the Michaelis-Menten equation to obtain K_m and V_{max} values (Table 5-1). The results were corrected for total protein concentration in each well. Each point represents the mean ± SD from three to four independent experiments performed in triplicates.

Table 5-1: Kinetic parameters of PFBS and PFHxS transport mediated by rat OAT1 and OAT3

Transporter	PFSA	K_m (µM)	V_{max} (nmol/mg protein*min^{-1})	V_{max}/K_m (µl/mg protein*min-^{1})
rOAT1	PFBS	285±60	2.8±0.3	9.8
	PFHxS	73±29	4.1±0.7	56
rOAT3	PFBS	57±17	1.6±0.2	28
	PFHxS	49±17	2.4±0.3	49

III. Discussion

In this chapter, we have demonstrated that rOAT1 and rOAT3 can transport PFBS and PFHxS, and determined the kinetic parameters of the transport.

Our results might help to explain the differences of elimination half-lives among PFSAs with different chain-lengths. Active secretion mediated by rOAT1 and rOAT3 of PFBS together with its relatively low molecular weight might contribute, at least in part, to the fast elimination in rats. The high molecular weight and serum protein binding prevent efficient glomerular filtration of PFHxS and PFOS. However, a small portion of PFHxS is excreted via urine, possibly due to the transport by rOAT1 and rOAT3. In the previous chapters, our results indicated that the liver transporters NTCP and OATPs are responsible for the uptake of PFBS, PFHxS and PFOS into hepatocytes. In general, PFOS and PFHxS were transported with higher efficiency than of PFBS, suggesting a greater probability for PFOS and PFHxS to accumulate in human and rat liver.

Our group characterized the transport of PFCAs with 7 and 8 carbons (C7 and C8) by rOAT1 and PFCAs with 8 and 9 carbons (C8 and C9) by rOAT3 in 2010 (Weaver et al., 2010). Compared to the transport of C7 (K_m=51 μM, V_{max}=2.2 nmol/mg protein*min^{-1}) and C8 (K_m=43 μM, V_{max}=2.6 nmol/mg protein*min^{-1}) by rOAT1, the transport of PFHxS (K_m=73 μM, V_{max}=4.1 nmol/mg protein*min^{-1}) has lower affinity but higher capacity, leading to a comparable overall intrinsic clearance.

The affinity of PFBS (K_m=57.0 µM for C8) and PFHxS (K_m=49 µM) transport is similar as for C8 (K_m=66 µM) transport by rOAT3 while the capacity is lower than the transport of both C8 (V_{max}=3.8 nmol/mg protein*min^{-1}) and C9 (V_{max}=8.7 nmol/mg protein*min^{-1}). Overall, for the transport by rOAT1 and rOAT3, PFHxS which corresponds in terms of carbon atoms to C7 and C8 have very similar characteristics.

In conclusion, our studies reveal that PFBS and PFHxS are substrates of rOAT1 and rOAT3, contributing to their relatively shorter elimination half-lives compared to longer chain-length PFSAs.

CHAPTER 6

Summary and overall discussion

The strong carbon-fluorine bonds of perfluoroalkyl substances (PFASs) make them excellent surfactants, but on the other hand, very resistant to environmental degradation. Consequently, once released into the environment, certain PFASs, including PFOA and PFOS, are very persistent and can be transported all around the world, even to the Arctic regions (Shoeib et al., 2006; Butt et al., 2010). PFOA and PFOS are detected in almost all the human blood samples being tested (Lau et al., 2007; Centers for Disease Control and Prevention, 2015). PFASs were also found in other human specimens, including umbilical cord blood (Apelberg et al., 2007), breast milk (Tao et al., 2008a; Tao et al., 2008b; Crinnion, 2010) and organs like liver, kidney and lung (Karrman et al., 2010; Perez et al., 2013). Based on epidemiological data obtained from retired workers, PFOA, PFHxS and PFOS have very long half-lives in humans (Olsen et al., 2007). Due to their wide distribution and extreme persistence, numerous studies focused on the toxicities and pharmacokinetics of PFOA and PFOS. However, a clear knowledge gap exists to explain the molecular mechanisms responsible for the long half-lives of PFOA, PFHxS and PFOS.

It was speculated that transporters as well as serum protein binding could contribute to the long elimination half-lives of PFOA and PFOS. Following this hypothesis, several studies regarding interactions between different serum proteins and PFOA and/or PFOS were conducted. The majority of the studies

dealt with interactions between PFOA and/or PFOS and serum albumin (Jones et al., 2003; Chen and Guo, 2009; Zhang et al., 2009; Salvalaglio et al., 2010; Luo et al., 2012; Beesoon and Martin, 2015; Wang et al., 2016a). In addition, PFOS and PFOA could also interact with hemoglobin, fatty-acid binding proteins as well as transthyretin-bound thyroxin in plasma (Luebker et al., 2002; Audet-Delage et al., 2013; Wang et al., 2016b). Furthermore, fluorescence displacement studies indicated that also the shorter chain length PFBS and PFBA could interact with human serum albumin (Chen and Guo, 2009). With respect to the role of membrane transport proteins in the pharmacokinetics of PFASs, previous studies mainly focused on PFCAs and kidney transporters. Weaver et al. and Yang et al. demonstrated that OATP1A1, which is expressed at higher levels in male rats, transports PFOA, potentially explaining the gender-dependent excretion of PFOA in rats (Yang et al., 2009; Weaver et al., 2010). In humans, OAT4 expressed at the apical membrane of proximal tubular epithelial cells mediates the reabsorption of PFOA from urine and therefore increases its elimination half-life (Yang et al., 2010). In contrast, little was known regarding the interaction of PFSAs and liver or intestinal transporters prior to this dissertation. Although Han et al. showed that active transport was involved in the uptake of PFOA into freshly isolated rat hepatocytes (Han et al., 2008), no specific transporter was previously identified.

In Chapters 3 and 4, I evaluated the hypothesis that liver and intestinal transporters involved in the enterohepatic circulation of bile acids contribute to

the long half-lives of PFHxS and PFOS in rats and humans. Because of the discontinuous sinusoidal capillaries present in the liver, even large molecules like serum proteins in the circulation can enter the perisinusoidal space surrounding the hepatocytes. This allows both unbound and bound PFSAs to access the space of Disse and to interact with transporters in the hepatocytes. The uptake system for organic solutes consists of sodium-dependent and sodium-independent transporters at the sinusoidal membrane of hepatocytes. Using cells overexpressing several of these different transporters, I demonstrated that both sodium-dependent and sodium-independent uptake transporters could mediate the transport of PFBS, PFHxS and PFOS in humans and rats. These transporters include human and rat NTCP, human OATP1B1, OATP1B3, OATP2B1, and rat OATP1A1, OATP1B2, and OATP2B1. Within the hepatocytes, PFOA and PFOS might bind to and be transported across the cell by fatty acid binding proteins (Luebker et al., 2002). My inhibition results obtained with Sf9 vesicles, shown in Chapter 3, suggest that after reaching the canalicular membrane, the human ABC transporters MRP2 and BCRP seem to be involved in the efflux of PFHxS while BSEP and MRP2 might handle PFOS. Along with bile, PFHxS and PFOS are released into the intestine after a meal. Transporters located at the brush border membrane of enterocytes could then reabsorb PFHxS and PFOS. In particular, I demonstrated that human ASBT mediates the influx of PFOS in a sodium-dependent manner. In contrast, PFBS, PFHxS and PFOS are substrates of human OATP2B1 and rat OATP1A5, indicating that these transporters play a role in the sodium-independent reabsorption of these

compounds from the intestine. Furthermore, I have also shown that OSTα-OSTβ transports PFBS, PFHxS and PFOS, suggesting a role for this heterodimeric transporter in the reabsorption of these compounds and their transport back into the blood stream. With the assistance of all these transporters, PFHxS and PFOS are retained in the body within the enterohepatic circulation for a long time, which is evidenced by the studies that showed that cholestyramine enhanced their fecal excretion (Johnson et al., 1984; Genuis et al., 2013).

In comparison with OATPs expressed in hepatocytes, the sodium dependent transporter NTCP is the dominant mediator for PFSAs transport, indicated by the hepatocyte uptake studies in Chapter 3. BSP inhibited both the sodium dependent and independent uptake of PFBS and PFHxS into human and rat hepatocytes. The fact that the BSP-inhibitable sodium independent uptake, which is the combination of the contribution of all OATPs, only constituted a minor portion of the total inhibition, suggests a major role of the sodium dependent transporter NTCP under the experimental conditions I used. The results regarding specific transporters in Chapter 3 and Chapter 4 are in good agreement with the uptake measurement obtained with isolated hepatocytes. Generally, in humans, the transport of the three PFSAs by OATPs has similar or even higher affinity compared to NTCP mediated transport. However, the capacity of PFSAs transport by NTCP (V_{max}=3.7 nmol/mg protein*min^{-1} for PFBS; 23.2nmol/mg protein*min^{-1} for PFHxS; 30.7 nmol/mg protein*min^{-1} for PFOS) is considerably higher than the one mediated by OATPs (V_{max}=0.2 to 0.6 nmol/mg

protein*min^{-1} for PFBS; 2.0 to 2.4 nmol/mg protein*min^{-1} for PFHxS; 0.8 to 4.7 nmol/mg protein*min^{-1} for PFOS). Taken together, NTCP-mediated uptake has a range of intrinsic clearance (V_{max}/K_m) of 100 to 200 µl/mg protein*min-^{1}, which is significantly higher than OATP-mediated transport. Regarding rat liver uptake transporters, I could not perform kinetic determinations for rat OATP1B2 and OATP2B1, as well as for PFOS transport by rat NTCP, due to the low signal-to-noise ratios. But the kinetic parameters for rat OATP1A1-mediated transport of PFHxS and PFOS, and rat NTCP-mediated transport of PFBS and PFHxS were determined. The affinities of rOATP1A1 and rNTCP were similar while rNTCP mediated uptake has a much higher capacity (V_{max}=8.1 nmol/mg protein*min^{-1} for rNTCP; 1.0 nmol/mg protein*min^{-1} for rOATP1A1). Collectively, the results of the hepatocyte uptake determinations as well as cell-based uptake studies for specific transporters indicate that NTCP plays a more important role than OATPs in mediating the influx of PFSAs into the liver.

Although OATPs are not the major players, it is still worth noticing that they are able to transport PFHxS and PFOS better than PFBS in humans and rats. The affinity of PFBS transport by OATPs is generally similar or a little bit lower than PFHxS and PFOS transport. However, the capacity of PFBS transport is much lower (V_{max}=0.2 nmol/mg protein*min^{-1} for human OATP1B1, OATP1B3 and rat OATP1A5; 0.6 nmol/mg protein*min^{-1} for human OATP2B1). As two liver-specific transporters, human OATP1B1 and OATP1B3 are frequently compared with each other with respect to substrate specificities. These two transporters share

many substrates, including estradiol-17β-glucuronide, BSP and several statins (Roth et al., 2012). However, they also have their specific substrates. For example, cholecystokinin octapeptide (CCK-8) is a substrate for OATP1B3 but not for OATP1B1 while estrone-3-sulfate at low concentrations is not transported by OATP1B3 (Hirano et al., 2004). When it comes to the transport of PFSAs, OATP1B1 and OATP1B3 behave very similarly for all three substrates in terms of affinity and capacity, as summarized in Table 4-3. Human OATP2B1 is not selectively expressed in the liver. A recent meta-analysis demonstrated that the expression level of OATP2B1 is close to OATP1B3 but lower than OATP1B1 in human liver (Badee et al., 2015). Table 4-3 shows that OATP2B1 transports PFBS and PFHxS similarly as OATP1B1 and OATP1B3 but it transports PFOS a little better than the other two transporters, indicated by the higher V_{max} value. In addition to PFSAs transport, uptake measurement of PFCAs with 7 to 10 carbons by human OATPs were also performed at one concentration and at one single time point. As PFOS (a C8 PFSA), PFOA (a C8 PFCA) is also a substrate for all the human liver OATPs. However, since we were not able to perform kinetic determinations, it is not feasible to compare PFOA and PFOS transport in detail.

PFBS, with only four carbon atoms, does not belong to the long chain PFSAs and is not as widely distributed as PFHxS and PFOS, possibly due to lower industrial production. However, it would be beneficial if we could understand the influence of transporters on the pharmacokinetics of PFBS because it has become one of the substitutes developed to replace PFOS. As PFHxS and

PFOS, PFBS is also a substrate of several transporters examined in the dissertation, including NTCP and all the OATPs. In addition, in Chapter 5, I demonstrated that rOAT1 and rOAT3 expressed at the apical membrane of proximal tubular epithelial cells mediates the transport of PFBS and PFHxS. Nevertheless, the estimated elimination half-life of PFBS (26 days in humans and 3.9-4.5 days in rats) is much shorter than the ones for PFHxS and PFOS and neither enterohepatic circulation nor liver accumulation have been reported for PFBS in humans or laboratory animals. By comparing the transport properties of PFBS to PFHxS and PFOS, we can explain the shorter half-life to some extent. Firstly, PFHxS and PFOS are better substrates of liver transporters NTCP and OATPs, indicated by the higher intrinsic clearance (V_{max}/K_m) listed in Table 3-2 and 4-3. Secondly, no inhibition of any efflux transporters by PFBS was seen, indicating that PFBS might not get into the enterohepatic circulation because it lacks a way out of hepatocytes into the bile ducts. Thirdly, although I showed that rOATP1A5 in the intestine can mediate the uptake of PFBS, its capacity to transport PFBS is much lower than the capacity for PFHxS and PFOS. So even if a small amount of PFBS reaches the intestine along with bile, it is likely to escape reabsorption. Last but not least, as we mentioned in Chapter 5, the smaller molecular size allows the majority of the dose of PFBS to undergo glomerular filtration in the kidney. With respect to transporters expressed in the proximal tubule, I have shown that rOATP1A1 is not a good transporter for PFBS while the transport by rOAT1 and rOAT3 is much better. Because of the potentially greater secretion than reabsorption, I conclude that PFBS is excreted

readily in urine, which can explain the much shorter elimination half-life in rats. In humans, PFBS is excreted faster than PFHxS and PFOS, but the half-life is still significantly longer than in rats. To address the mechanisms for the species difference, additional tests are needed. For instance, it is not known whether PFBS is a substrate of hOAT4, which could influence its renal excretion since the transporter is expressed at the apical membrane and mediates the reabsorption of organic anions in the proximal tubule in humans.

Among all the transporters tested, only OATP1B1 and OATP1B3 are selectively expressed in human liver. The other transporters are expressed in additional cells beside hepatocytes and/or enterocytes. In addition to the enterohepatic circulation, ASBT expressed at the apical membrane of bile duct epithelial cells also participates in the formation of the intrahepatic circulation. As a substrate of human ASBT, PFOS might get trapped within the liver via this intrahepatic circulation. OATP2B1, which can mediate the influx of PFOS, is also located at the blood brain barrier (BBB) and blood placenta barrier (Roth et al., 2012). However, unlike in the liver, the concentration of PFOS in cerebrospinal fluid is considerably lower than in serum and no accumulation has been seen (Harada et al., 2007). The underlying mechanism might include binding of PFOS to plasma membrane proteins to prevent it from crossing the tight BBB. Besides this, there might be efflux pumps like MRP3 located at the BBB that mediate the extrusion of PFOS, which have not been tested yet (Zhao et al., 2015b). Furthermore, as I mentioned before, PFOS is found in umbilical cord blood, suggesting potential

placental transfer in humans. The involvement of transporters in mediating the maternal to fetal transport has not been reported so far. However, there is evidence showing that human OAT4, expressed at the fetal side of the epithelium, might reduce the placental passage of PFOS and PFOA (Yang et al., 2010; Kummu et al., 2015). Like OAT4, OATP2B1 is also expressed at the fetal side in the placenta, suggesting it might also play a role in decreasing the placental transfer of PFOS.

As shown in Chapters 3 to 5, the background uptake increases as the chain-length increases. One reason could be non-specific binding to the membrane due to increased lipophilicity. To assay this, vesicles made from CHO or HEK293 cells could be used to confirm whether the chemicals get into cells or stay in the membrane. Another possibility for the high background is the expression of other so far not identified transporters, which can transport PFOS, in these cell lines.

Until now, studies of PFASs in humans have been mainly focused on plasma concentrations. However, the knowledge on the burden of PFASs in other human compartments is also important for monitoring as well as for health risk assessment. A physiologically based pharmacokinetic (PBPK) model in monkeys for PFASs was first generated to fill in this knowledge gap (Andersen et al., 2006). The authors claimed that capacity-limited, saturable processes must be involved in the kinetic behavior of PFOA and PFOS because they found that the apparent elimination rates differ with increasing dose. Therefore, renal reabsorption by

transporters should be considered. Later, another group included renal absorption for PFOA and PFOS to their PBPK model in humans and rats, and was able to successfully simulate the data collected from residents of two communities exposed to PFOA in drinking water (Loccisano et al., 2011; Loccisano et al., 2012). Kinetic parameters of PFOA transport by OATP1A1 calculated by Yang, et al. (Yang et al., 2009) were used in the rat PBPK model. In these models, uptake of PFOA and PFOS into the liver was considered a simple diffusion process. However, my findings suggest that it is a saturable process, suggesting the involvement of carrier-mediated transport. Thus, it is reasonable to speculate that the results in my dissertation could help to improve the PBPK model in rats and humans, especially for the liver compartment.

Overall, in this dissertation, I examined the interactions of PFASs with membrane transport proteins, focusing on the liver, intestine and kidney. First of all, I demonstrated that the uptake of PFBS, PFHxS and PFOS into freshly isolated human and rat hepatocytes involves active transport. Based on this result, I found that transporters expressed within the enterohepatic circulation mediate the transport of PFBS, PFHxS and/or PFOS. In the liver, human and rat NTCP, human OATP1B1, OATP1B3, OATP2B1 and rat OATP1A1, OATP1B2, OATP2B1 can mediate the transport of all three PFSAs. At the canalicular membrane of human hepatocytes, PFHxS and PFOS inhibited efflux transporters for organic anions. In the intestine, I showed that human ASBT can mediate the reabsorption of PFOS while all three PFSAs are substrates of human OATP2B1

and rat OATP1A5. Human OSTα-OSTβ, which mediates the influx and efflux of substrates along their concentration gradients, could transport PFBS, PFHxS and PFOS. In addition, I found that rat OAT1 and rat OAT3 expressed in the rat kidney can transport PFBS and PFHxS and thus could facilitate their renal secretion. However, we still need to prove these interactions *in vivo* and figure out a way to extrapolate the *in vitro* kinetic values to the *in vivo* conditions.

CHAPTER 7

Future directions

The results in this dissertation were all generated based on *in vitro* systems. How to confirm them *in vivo* becomes a major challenge, not only for the dissertation, but also for transport studies in general. Common resources used for uptake studies include perfused organs, isolated cells, isolated membrane vesicles, as well as cloned specific transporters that are expressed in oocytes or mammalian cell lines (Hagenbuch, 2010). Nowadays, cell-based transport assays have become very popular because on the one hand, specific transporters can be identified and on the other hand, they are more convenient for kinetic determinations. Using these methods, many substrates of SLC transporters have been identified. In order to prove that the transport really occurs *in vivo*, specific or selective inhibitors of the tested transporters should be used for these examinations. Although it is not feasible to perform such tests in humans, using freshly isolated hepatocytes is the gold standard alternative for human liver studies. However, to the best of our knowledge, selective inhibitors for OATPs or NTCP are not yet available. Even if we could find such inhibitors, the results might not allow to make final conclusions because of the redundancy of several transporters for one compound in the same membrane. For example, selective inhibitors of ASBT, such as A4250 (Baghdasaryan et al., 2016), were developed because of their potential therapeutic effect. However, even when ASBT is inhibited, OATP2B1 in the intestine can still mediate the reabsorption of PFOS in humans. Therefore, the lack of selective inhibitors for these human transporters

together with the redundancy of several transporters for one substrate makes it difficult to address the *in vivo* situation. Besides human transporters, rat transporters were also included in the dissertation and in many other studies. As for human transporters, no specific inhibitors are available for the rat transporters. Knockout rats for any of these transporters are not available so far. However, with the development of zinc finger nuclease technology (Geurts et al., 2009) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system (Shao et al., 2014), it should be possible to obtain knockout rats for these transporters. However, again, due to the fact of several transporters for one substrate, single gene knockout animals can easily lead to redundancy and compensation. One potential solution is to generate conditional double knockout rats with inactivated NTCP-and OATP1A/1B, for instance, in the liver, to confirm their roles *in vivo*. Although Oatp1a/1b-knockout mice are available (van de Steeg et al., 2010), transport of PFASs by mouse transporters has not been determined yet. Given the similarity of transporting properties between mouse and rat transporters, we might speculate that the same mouse transporters are involved in the disposition of PFASs as in rats. However, compensation by NTCP in the liver and ASBT in the intestine might still exist.

To better address the mechanisms for the long half-lives of PFOA and PFOS with respect to drug transporters, it is also important to study the efflux transporters. In this dissertation, I only conducted inhibition studies for the three human efflux transporters BSEP, BCRP and MRP2, using Sf9 vesicles. However,

direct transport measurements are needed since inhibition does not necessarily mean that the inhibitors are also substrates. Several methods could be adopted in order to determine direct transport in the future. For instance, sandwich cultured hepatocytes are a powerful tool to measure hepatobiliary drug transport (Swift et al., 2010). Biliary efflux studies could be conducted by including steps to preload the cells, disrupt the tight junctions and then incubate the cells with their respective buffers as described earlier (Liu et al., 1999). Although this method is suitable to demonstrate biliary transport, specific transporters cannot be distinguished. By expressing a pair of uptake and efflux transporters in a polarized cell line, like the Madin-Darby canine kidney (MDCK) cells or the porcine kidney LLC-PK1 cells, vectorial transport could be measured to identify a substrate of either the uptake or the efflux transporters. If we use this method for PFSAs, NTCP could be expressed at the basolateral membrane as an influx mediator and at the apical membrane, the efflux transporter of interest (BSEP/BCRP/MRP2) could be expressed. In this way, a compound could be tested by measuring trans-cellular transport.

This dissertation focused on PFSAs transport by liver and intestinal transporters. With respect to kidney transporters, I determined the transport of PFBS, PFHxS and PFOS by rat OAT1, OAT3 and OATP1A1, which are also transporters for PFCAs. Nevertheless, it is still important to examine the human kidney transporters for PFSAs because renal reabsorption could contribute to the long half-life of a compound. Human OAT4 and URAT1 were shown to transport

PFOA. Since PFOS is structurally very similar to PFOA, these two transporters are good candidates to be tested in the future.

Given that PFOS and PFHxS excretion is enhanced by cholestyramine treatment PFOA might also undergo enterohepatic circulation in rats and humans (Johnson et al., 1984; Genuis et al., 2013). However, previous studies of PFOA have focused only on kidney transporters. Sodium-dependent transport of PFSAs was first reported in my dissertation and NTCP seems to transport PFSAs better than OATPs. In the future, it would be interesting to test whether the same transporters that are involved in the enterohepatic circulation of PFSAs are also involved in the enterohepatic circulation of PFOA, especially the sodium dependent transporters NTCP and ASBT.

All the experiments and strategies mentioned above will lead to a better understanding of the molecular mechanisms of the long half-lives of long-chain PFASs in humans and should allow to improve existing PBPK models that can be used to predict the disposition of these environmentally persistent compounds. Furthermore, this work will help to identify individuals who might be susceptible to the potential adverse effects of PFASs. For instance, occupational workers with OATP1B1/OATP1B3 polymorphisms that result in high transporter expression could have much higher concentrations of these compounds in the hepatocytes and potentially lead to liver diseases, such as hepatomegaly, steatosis, and hepatocellular carcinoma via activation of HNF4α.

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